Identification of Cancer Stem Cells in Malignant Pleural Mesothelioma

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von

Claudia Ursula Frei

von Hagenbuch ZH

Promotionskomitee

Prof. Dr. Lukas Sommer (Vorsitz der Dissertation)

Prof. Dr. med. Rolf A. Stahel

PD Dr. Emanuela Felley-Bosco (Leiterin der Dissertation)

Prof. Dr. Massimo Lopes

Prof. Dr. Gerhard Christofori

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1 ZUSAMMENFASSUNG

In der Krebsforschung herrscht heutzutage die Ansicht vor, dass das Tumorwachstum, Tumorrezidive sowie die Entwicklung von Chemoresistenzen auf Krebsstammzellen zurückzuführen sind. Diese Krebsstammzellen sollen ihre Eigenschaften mit jenen adulter Stammzellen gemein haben, wie es für die akute myeloische Leukämie gezeigt wurde. Krebsstammzellen solider Tumoren werden, wenn immer möglich, anhand von Zelloberflächenmarkern identifiziert, welche für die adulten Stammzellen des betroffenen Gewebes bekannt sind. Diese Vorgehensweise ist nicht möglich wenn Oberflächenmarker adulter Stammzellen nicht bekannt sind. In solchen Spezialfällen wird eine funktionelle Methode zur Identifizierung der Krebsstammzellen namens „side population“ (SP)-Methode verwendet.

Das Ziel dieser Forschungsarbeit war ursprünglich die Identifizierung von Krebsstammzellen des malignen Pleuramesothelioms (MPM) anhand dieser funktionellen Methode. Das maligne Pleuramesotheliom ist ein Tumor, welcher von Mesothelzellen ausgeht, nachdem sich Asbestfasern in der Pleura, welche die Lunge umschliesst, anreicherten. Dieser Tumor ist sehr bösartig und weisst eine mittlere Überlebensrate von einem Jahr ab Diagnose auf. Zudem macht sein schlechtes Ansprechen auf die Chemotherapie sowie sein zunehmendes Auftreten notwendig mehr über seine Entstehungsbiologie zu erfahren.

Das Datenmaterial dieser Forschungsarbeit zeigt die Existenz von SP-Zellen im MPM auf. Wir konnten *in vitro* zeigen, dass SP-Zellen des Pleuramesothelioms Eigenschaften zur Selbsterneuerung sowie Ausdifferenzierung aufweisen, wie es das Krebsstammzellen-Modell besagt. In Xenotransplantationsexperimenten in Mäusen beobachten wir die Tendenz von SP Zellen zu grösserer Tumorentwicklung verglichen mit nicht-SP (NSP)-Zellen. Tumore, welche sich von SP-Zellen entwickelten, wiesen einen erhöhten Anteil an sarcomatoiden MPM auf. Dieser Sub-Typ ist der aggressivere der MPM Sub-Typen und ähnelt einem Vorläuferzellen-Phänotyp. Ein erhöhter Anteil an sarcomatoiden Mesotheliomzellen wurde auch in wiederkehrenden Tumoren von Patienten festgestellt. Zudem konnten wir zeigen, dass MPM-SP-Zellen im Vergleich zu NSP-Zellen resistenter gegenüber Chemotherapeutika waren.

Zusammenfassend haben wir im malignen Pleuramesotheliom Zellen identifiziert, welche selbsterneuernde Eigenschaften *in vitro*, die Tendenz erhöhter Tumorentwicklung *in vivo,* sowie erhöhte Chemoresistenz aufweisen. Zusätzlich beobachten

wir, dass Tumore, welche von MPM-SP-Zellen hervorgehen, Merkmale aufweisen, welche auch bei wiederkehrenden, sarcomatoiden Tumoren von MPM-Patienten beobachtet wurden.

2 SUMMARY

Recently it has been hypothesized that tumor development, recurrence and chemoresistance are due to cancer stem cells (CSCs). The latter may share properties of tissue stem cells, as demonstrated for acute myeloid leukemia (AML). In solid tumors, where possible, CSCs are identified according to the expression of known stem cell surface markers of the tissue of origin. Where this approach is not possible due to the lack of known stem cell surface markers, a functional assay called side population (SP) assay is used.

The aim of this study was originally to use such an approach to identify cancer stem cells of malignant pleural mesothelioma (MPM). Malignant pleural mesothelioma is a tumor of mesothelial origin developing after exposure to asbestos fibers in the pleura, which surrounds the lung. Due to its severity with a survival rate of around one year after diagnosis, the development of chemoresistance and the increasing incidence, it is of great importance to gain more insight into the biology of MPM.

The data obtained in this study revealed the presence of SP cells in MPM. As the CSC model claims, these SP cells have self-renewal and differentiation capacity, which was observed for MPM SP cells *in vitro*. In a xenograft mouse model we observed the tendency of SP cells to be more tumorigenic *in vivo* compared to non-side population (NSP) cells. Tumors developed from SP cells contained cells with an increased spindeloid morphology. The latter is a more severe subtype of MPM and may represent a precursor phenotype. The increase of sarcomatoid histotype was also observed in patients with recurring MPM. Additionally it could be observed that the chemoresistance of SP cells was increased compared to NSP cells.

In summary we identified in MPM a subset of cells with self-renewal capacity *in vitro*, tendency of increased tumorigenicity *in vivo*, with increased chemoresistance and which developed tumors mimicking recurrence tumors.

3 INTRODUCTION

3.1 MALIGNANT PLEURAL MESOTHELIOMA

Malignant pleural mesothelioma is the most common neoplasm of the mesothelium, which is the lining of several body cavities such as the pleura, pericardium and the peritoneum as well as the tunica vaginalis[1] (Mutsaers and Wilkosz, 2007). Malignant pleural mesothelioma is associated with exposure to asbestos fibers (Wagner et al., 1960). Exposure occurs either occupationally, para-occupationally (i.e. mine worker's wives), or non-occupationally (i.e. environmentally) (Moore et al., 2008). In very rare cases it occurs spontaneously (Moore et al., 2008).

It is a devastating disease with a survival rate of around 12 months after diagnosis (Stahel and Weder, 2009). Despite asbestos ban in the 1990s, its incidence is still rising with an expected peak in 2020 (Peto et al., 1999).

3.1.1 ANATOMY OF THE PLEURA

The pleura is a serous membrane surrounding the lung. It is subdivided into the visceral (inner) pleura directly adjoining the lung and the parietal (outer) pleura covering the chest wall including the diaphragm and the mediastinum (Figure 1) (Mutsaers and Wilkosz, 2007). The space between the visceral and parietal pleura is the pleural space; it is enclosed and contains pleural fluid composed of glycoprotein-rich fluid and has a low cell count containing rare macrophages, mesothelial cells and lymphocytes (Murali et al., 2010). The composition of the visceral and parietal pleura includes five layers starting from the pleural surface: the mesothelium (Figure 2, no. 1), a thin layer of submesothelial connective tissue (Figure 2, no. 2), a superficial elastic tissue layer (Figure 2, no. 3), a second loose sub-pleural connective tissue layer rich in arteries, veins, nerves and lymphatics (Figure 2, no. 4) and a deep fibroelastic layer adherent to the underlying lung parenchyma, chest wall, diaphragm or mediastinum (Figure 2, no. 5) (Wang, 1998).

[1] Serous covering of the testis

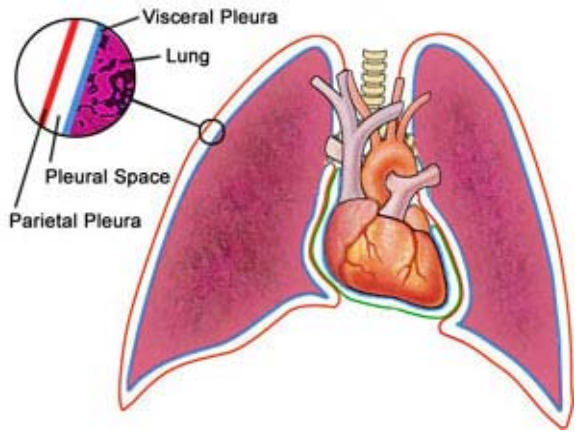

Figure 1: Anatomy of the pleura. Adapted from www.virtualmedicalcenter.com.

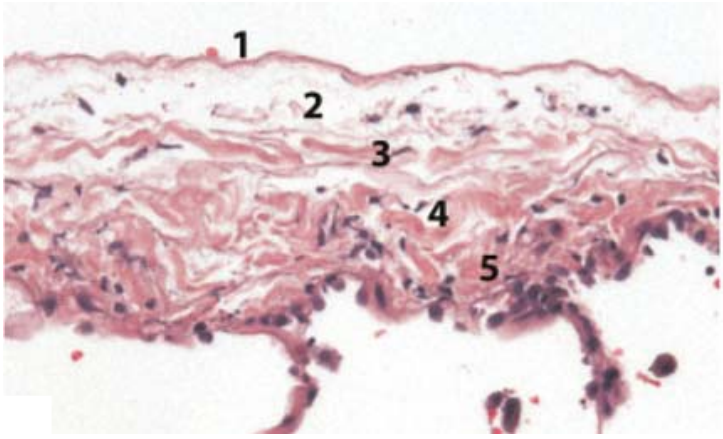

Figure 2: The five layers of the pleura. A histological view. Adapted from Murali, 2010.

3.1.2 THE MESOTHELIUM

The mesothelium is composed of specialized mesothelial cells forming a monolayer. Fully differentiated mesothelial cells (25 μm in diameter) are predominantly flattened, squamous-like cells (Mutsaers and Wilkosz, 2007) with microvilli at their luminal surface, to enlarge the cell surface area of secreting/absorbing cells (Murali et al., 2010). Mesothelial cells show denser distribution of microvilli at the visceral pleura and a more sparse density at the parietal pleura (Murali et al., 2010).

Mesothelial cells have epithelial as well as mesenchymal characteristics (LaRocca and Rheinwald, 1984, Herrick and Mutsaers, 2004) such as the expression of epithelial marker cytokeratin, polygonal cell shape, the secretion of basement membrane and the expression of mesenchymal vimentin and desmin (Herrick and Mutsaers, 2004). Upon tissue injury mesothelial cells are activated to proliferate extensively to repair the lesion (reviewed by (Herrick and Mutsaers, 2004)) and the existence of

rare cells with progenitor-cell characteristics within normal mesothelial cells have recently been described (Lansley et al., 2010).

3.1.3 Pleural Embryogenesis

Mesothelial cells derive from embryonic mesodermal cells, where they develop from the coelomic cavity during early embryogenesis (Murali et al., 2010). From the neural groove the intraembryonic mesoderm differentiates on each side into paraxial, intermediate and lateral mesoderm (Herrick and Mutsaers, 2004). Later the intraembryonic somatic or parietal layer and the intraembryonic splanchnic or visceral layer form, which takes place after spaces appear in the lateral mesoderm fused and divided the mesoderm in these two layers (Herrick and Mutsaers, 2004). Next the somatopleure (embryonic body wall) is formed by the somatic mesoderm together with the embryonic ectoderm (Herrick and Mutsaers, 2004). On the other hand the splanchnopleure (embryonic gut wall) is formed by the splanchnic mesoderm together with the embryonic ectoderm (Herrick and Mutsaers, 2004). By extending the somatopleure as well as the splanchnopleure along the embryonic length and fusing ventrally creates the intraembryonic coelum which represents a primitive body cavity (Wang, 1998). Later the splanchnopleure will form the viscera and heart as well as the visceral pleura (Herrick and Mutsaers, 2004), (Wang, 1998). The somatopleure will form the body wall lining and dermis as well as the parietal pleura (Herrick and Mutsaers, 2004), (Wang, 1998).

It was shown that epithelial cells of the coelomic wall express podoplanin which later line the pleural cavity (Gittenberger-de Groot et al., 2007), (Mahtab et al., 2008). Podoplanin expression is important during embryogenesis, because it was observed that knocking down podoplanin in mouse embryos lead to embryonic and fetal death at stages E10 – E16 as well as to neonatal death within the first weeks of life (Mahtab et al., 2008). Podoplanin expression in peritoneal mesothelial cells was for the first time observed in 1997 (BreitenederGeleff et al., 1997). Hence podoplanin might represent a marker for the coelomic mesothelium, which is maintained in some adult mesothelial cells with precursor properties.

Additionally, coelomic epithelium during embryogenesis also expresses Wilms tumor gene *WT1* (summarized by (Moore et al., 1999)). It is suggested that WT1 plays a role in epithelial-to-mesenchymal transition (EMT) during embryogenesis (summarized by (Moore et al., 1999)) since down-regulation of WT1 is associated with EMT

(Wilm et al., 2005). The first WT1 expression during embryogenesis is observed at E9 (days post coitum)[2] in the region of developing heart (Moore et al., 1999) and WT1 null mice die with malformed heart at midgestation (Moore et al., 1999). WT1 is then expressed throughout embryogenesis and adulthood in the serosal mesothelium (Wilm et al., 2005).

3.1.4 FUNCTION OF THE PLEURA

The mesothelium provides many different, essential functions. By secreting glycosaminoglycans (GAGs) it provides a slippery surface to minimize the friction during breathing which is essential for the lung to work properly. The amount of this so called pleural fluid in healthy tissues is around 0.1 – 0.2 ml/kg body weight (Murali et al., 2010). For the homeostasis of the pleural fluid, the lymphatic system is of great importance. In the caudal portion of the parietal pleura, pores called stomata, connect the pleural space to the lymph, allowing accumulating fluid and inflammatory cells to be removed into the lymphatic system (English and Leslie, 2006).

The slippery surface also protects the pleura from infections as well as tumor dissemination (Herrick and Mutsaers, 2004), most probably in a way that cells, bacteria do not adhere to the mesothelium. Additionally, mesothelial cells secret various mediators (i.e. growth factors and cytokines) to modulate and participate inflammation and which help trafficking leukocytes across the pleural space (Herrick and Mutsaers, 2004). Furthermore, upon stimuli like tissue injury, asbestos and bacterial products, mesothelial cells produce different cytokines resulting in a pro- or anti-inflammatory response helping to regulate the inflammatory response. Additionally, growth factors produced by mesothelial cells help to stimulate cell proliferation, differentiation and migration of themselves to sides of tissue injury therefore stimulating tissue repair (Herrick and Mutsaers, 2004).

3.1.5 ASBESTOS

Asbestos is a naturally occurring silicate mineral fiber extremely heat persistent and flexible (Yang et al., 2008), (Donaldson et al., 2010), (Donaldson, 2009). Therefore it was used due to its softness and flexibility in many fields to make each material more fire persistent (i.e. curtains, fire protecting cloths, in brakes as well as in military masks) (Alleman and Mossman, 1997), (Yang et al., 2008).

[2] Age of an embryo

Asbestos is divided into two forms: the serpentines (including chrysotile, the white asbestos, Figure 3) and amphiboles (including crocidolite, the blue asbestos and amosite, the brown asbestos, Figure 3). Crocidolite and amosite are the most common amphiboles with crocidolite to be the most dangerous form due to its long and thin fiber shape. Nevertheless, chrysotile was the form mostly in use (95% of used asbestos in the world). It is not as dangerous as amphiboles due to its softer structure which can be broken down by the body.

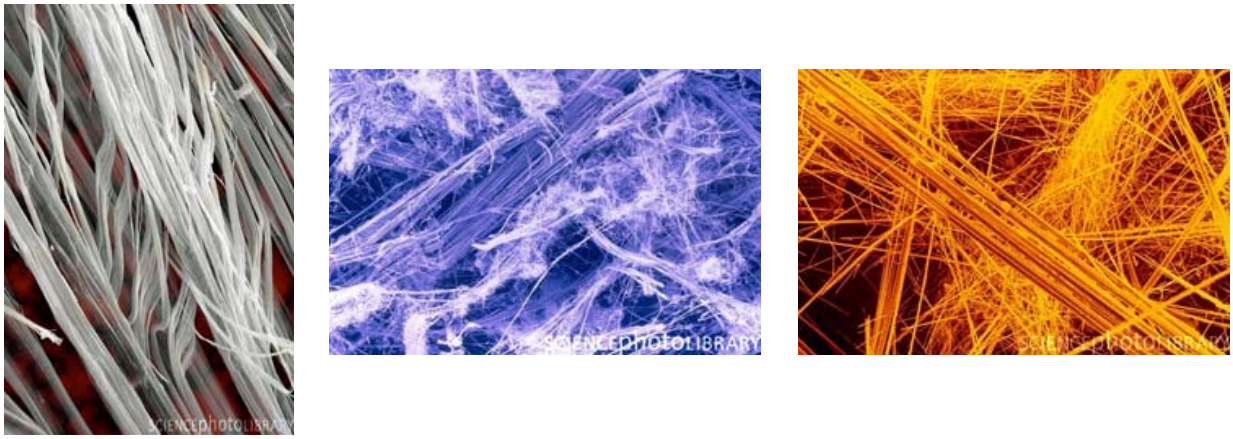

Figure 3: Asbestos fibers representing chrysotile (white), crocidolite (blue), amosite (brown) asbestos. Science photo library, Dr. Jeremy Burgess and Power and Syred, (www.sciencephoto.com).

3.1.6 ASBESTOS CLEARANCE

Dependent on its aerodynamic diameter, inhaled particles can be cleared from the respiratory tract at different deposition sites (Lippmann et al., 1980). Soluble particles are most likely taken up systemically at all deposition sites (Lippmann et al., 1980). Particles which are slowly soluble are transported from the deposition site in the conducting airways to the glottis[3] to be swallowed (Lippmann et al., 1980). Insoluble particles with large surface-to-volume ratios can deposit in non-ciliated airways and may be cleared by dissolution (Lippmann et al., 1980). Additionally they can be cleared as free particles or they are phagocytosed and transported by alveolar macrophages (Lippmann et al., 1980). Particles reaching the epithelium either as free particles or

[3] Combination of the vocal folds and the space in between the folds

within macrophages are either discharged within cells or might reach the pleura as well as lymph nodes (Lippmann et al., 1980).

The toxic characteristics of inhaled fibers is most importantly their geometry not their chemical composition (Donaldson et al., 2010). Chemical composition only plays a role concerning its biopersistence (Donaldson et al., 2010). Therefore, the fiber pathogenicity paradigm claims that a long, thin and biopersistent fiber is pathogenic (Donaldson et al., 2010). Because the fibers are so long macrophages undergo frustrated phagocytosis, a process in which macrophages fail to completely enclose long fibers (Figure 4) (Donaldson et al., 2010). Frustrated phagocytosis is a pro-inflammatory process releasing oxidants and cytokines and therefore probably participates in mesothelioma development.

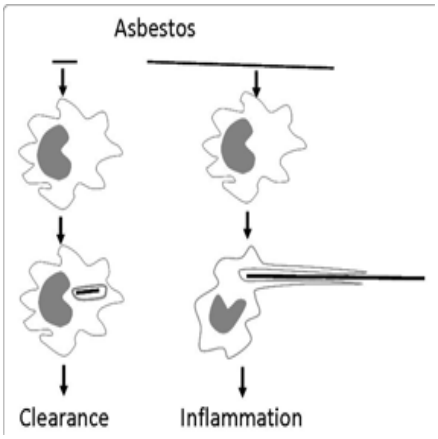

Figure 4: Illustration of frustrated phagocytosis, right panel leading to inflammation. Left panel illustrates normal phagocytosis leading to clearance. Adapted from Donaldson, 2010.

Furthermore, asbestos fibers reaching the pleura as free particles might depose at parietal stomata (pore like structure < 10μm), because they are too long to pass through to reach the lymph system (illustrated in Figure 5, (Donaldson et al., 2010)). Accumulated asbestos fibers at the stomata attract macrophages (Yang et al., 2008) leading to frustrated phagocytosis (Donaldson et al., 2010).

Nevertheless, as Cugell already stated: “how asbestos fibres…migrate to the pleura surface…is quite obscure.” (Cugell and Kamp, 2004).

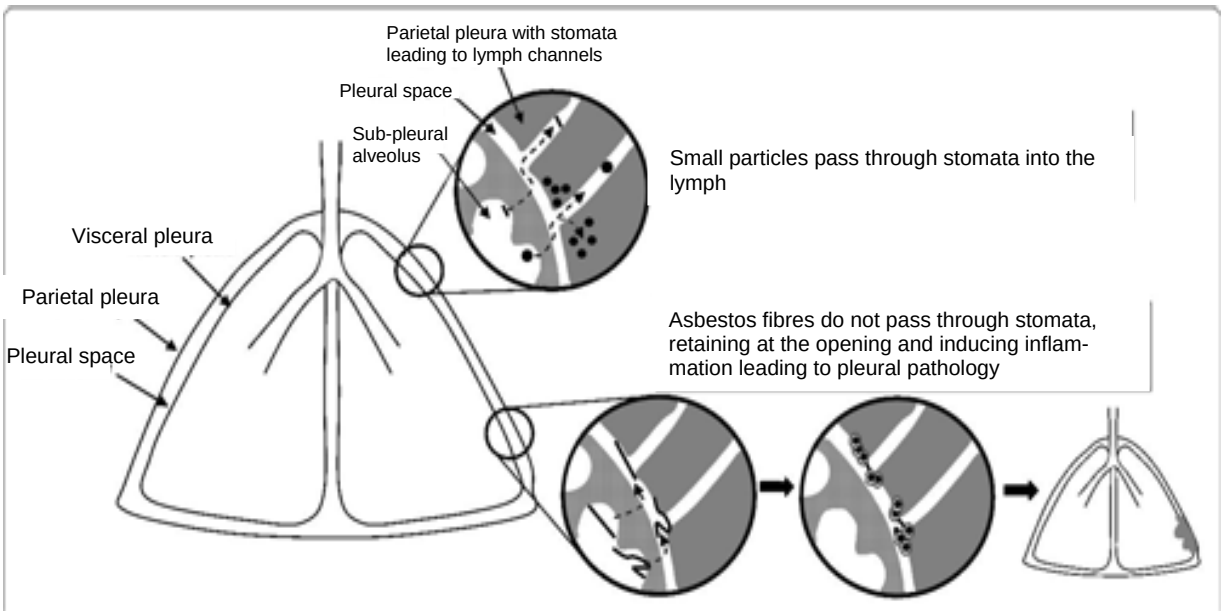

Figure 5: Hypothesized asbestos fibers retention at stomata leading to inflammation. Adapted from Donaldson, 2010.

3.1.7 MESOTHELIOMA CARCINOGENESIS

Upon asbestos deposition in the pleura and frustrated phagocytosis, macrophages release tumor necrosis factor-alpha (TNF-α) (Yang et al., 2008). Simultaneously, it initiates mesothelial cells to express TNF-R1, the TNF-α receptor at its cell membrane (Yang et al., 2008). Additionally, mesothelial cells start to release TNF-α in a paracrine and autocrine manner (Yang et al., 2008). TNF-α binding to its receptor expressed by mesothelial cells, induces the NF-κB pathway resulting in a better survival of mesothelial cells upon asbestos exposure (Yang et al., 2008). Also other growth factors and cytokines are released like transforming growth factor beta (TGF-β), insulin-like growth factor (IGF), interleukins 6 and 8 (IL-6/-8) and some more (Yang et al., 2008), (Yao et al., 2010).

Furthermore, asbestos fibers (with a certain iron content) induce DNA damage in mesothelial cells via free radicals (iron-catalyzed) which produce DNA strand breaks (Cugell and Kamp, 2004), (Yang et al., 2008). Further, mutations like base substitutions, deletions and rearrangements and sister chromatin exchange are caused by reactive oxygen species (ROS) and/or reactive nitrogen species (RNS) released upon asbestos exposure (Yang et al., 2008). The increased survival of mesothelial cells induced by TNF-α and the NF-κB pathway in combination with accumulated DNA

damages leads to carcinogenic transformations of mesothelial cells and further to mesothelioma (Yang et al., 2008), (Yao et al., 2010).

Upon mutations caused by asbestos fibers, chromosomal regions harboring tumor suppressor genes are deleted or inactivated. In mesothelioma the most commonly observed chromosomal change (40%) takes place on chromosome 22q12 where the neurofibromatosis type 2 (*NF2)* gene is located and which encodes the tumor suppressor merlin (Thurneysen et al., 2009). *NF2* is thought to be a 'gatekeeper' in mesothelioma induced by asbestos, because it was shown that upon deletion of one allele in mice, the frequency of tumor development after asbestos exposure increased compared to wild–type mice (reviewed by (Stahel et al., 2009)) and additionally these mice lost their second allele of *NF2* (reviewed by (Stahel et al., 2009). A second region also often deleted or inactivated in mesothelioma locates at chromosome 9p21 including the gene *INK4α/ARF* which encodes p16(INK4A) and p14(ARF) (Stahel et al., 2009), (Yang et al., 2008). *P53* deletion or inactivation is only occasionally observed in mesothelioma (Yang et al., 2008).

Deletion and/or inactivation of *NF2* increases cyclin D1 expression driving cells to enter S-phase (Xiao et al., 2005) and which leads to increased proliferation. Deletion and/or inactivation of p16(INK4a) encoding a cyclin-dependent kinas inhibitor, and p14(ARF), encoding a component of the p53 cell cycle checkpoint, results in impaired retinoblastoma (Rb) and p53 pathways (Yang et al., 2008).

3.1.8 DIAGNOSIS OF MALIGNANT PLEURAL MESOTHELIOMA

Due to its long latency time, patients most commonly are 50 to 70 years old. They suffer from dyspnea[4], coughing and chest pain or both (Moore et al., 2008). In rare cases sweating, weight loss and chest wall mass occur (Moore et al., 2008). Further signs of mesothelioma are pleural effusion, fatigue, wheezing, blood in the fluid coughed up, pneumothorax[5] or even collapse of the lung. In a later stage also dysphagia[6] occurs (Moore et al., 2008).

For diagnosis and staging of mesothelioma radiological and pathological technics are used. Radiology includes X-ray, computer tomography (CT), magnetic resonance imaging (MRI) and positron emission tomography (PET) (Moore et al., 2008). The radiological technique which is used first in MPM diagnosis is the intravenous con-

[4] Shortage of breath
[5] Collection of air in the pleural cavity
[6] Symptom of difficulty in swallowing

trast-enhanced CT scan (Moore et al., 2008). It has the advantage of visualizing the whole pleural surface including the diaphragm (Moore et al., 2008). CT scans are helpful in diagnosing MPM with results as 1) a circumferential pleural rind, 2) nodular pleural thickening, 3) pleural thickening of > 1 cm and 4) mediastinal pleural involvement (Moore et al., 2008). Nevertheless, absence of these findings does not exclude diagnosis of mesothelioma (Moore et al., 2008).

MRI for mesothelioma diagnosis is not routinely used (Moore et al., 2008). It is helpful to investigate tumor spreads into the diaphragm or chest wall to make better estimation before surgery (Moore et al., 2008).

PET scans are used to distinguish benign from malign pleural diseases (Moore et al., 2008). Therefore a sub-variant of PET, the standardized uptake value (SUV) PET is used, because it is a semi-quantitative measurement of a lesion's metabolic activity which is higher in mesotheliomas than in benign pleural diseases (Moore et al., 2008). Due to its metabolic and anatomical information PET scans are helpful for staging mesothelioma (Moore et al., 2008).

Diagnosis and staging should not only rely on different radiological methods but should be combined with pathological investigations (van Meerbeeck et al., 2011). Ideally a biopsy specimen of MPM should be used and investigated by immunohistochemistry and microscopy (van Meerbeeck et al., 2011). Best immunohistochemical markers to identify MPM are cytokeratin 5/6, calretinin, podoplanin and WT1 (Husain et al., 2009), (Mutsaers, 2002). It is also recommended to include negative markers. To distinguish MPM from other malignancies of the thorax like lung adenocarcinoma and squamous carcinoma markers which are differentially expressed between the malignancies are used such as MOC-31 and CEA for lung adenocarcinoma or p63 and MOC-31 for squamous carcinoma (Husain et al., 2009).

A very crucial issue in diagnosing and staging mesothelioma is the identification of mesothelioma's histotype (Husain et al., 2009). Mesothelioma can be sub-divided into epithelioid, sarcomatoid and biphasic mesothelioma (Husain et al., 2009). The histology of epithelioid MPM includes cells of cuboidal, polygonal or oval shape mimicking normal mesothelial cells (Husain et al., 2009). On the other hand, the histology of sarcomatoid MPM includes cells of spindle like morphology (Husain et al., 2009). The biphasic histotype includes epithelioid as well as sarcomatoid parts in the same tumor (Husain et al., 2009). Patients diagnosed with sarcomatoid mesothelioma have

worst prognosis (Belli et al., 2009), (Stahel and Weder, 2009), (Sugarbaker and Norberto, 1998).

Staging after diagnosis should be done according to the international mesothelioma interest group (IMIG) TNM staging system (Appendix 1, (Moore et al., 2008), www.imig.org).

3.1.9 TREATMENT OF MALIGNANT PLEURAL MESOTHELIOMA

In general the treatment of patients diagnosed with malignant pleural mesothelioma is controversial and depending on the fitness of a patient to undergo surgery.

Surgery, even though controversial for MPM treatment (Kaufman and Flores, 2011), is aimed at resection the tumor bulk as well as for diagnostic purposes. To achieve a biopsy for diagnosis a thoracoscopy is performed (Stahel and Weder, 2009). Further, for resection of tumor bulk either pleurectomy/decortication or a more radical method extrapleural pneumonectomy (EPP) is used (Stahel and Weder, 2009). Still debates are ongoing which method is the best for resection (Stahel and Weder, 2009). The milder pleurectomy/decortication (P/D) involves the removal of the parietal pleura including parts of the mediastinum, pericardium and diaphragm together with decortication of the lung by stripping off the visceral pleura (Ismail-Khan et al., 2006). Unfortunately in almost 80% of P/D, small tumor bulk is left in leading to recurrence in up to 90% of cases (reviewed by (Ismail-Khan et al., 2006). EPP on the other hand is a more radical method which removes altogether the visceral and parietal pleura together with the affected lung diaphragm, pericardium and mediastinal lymph nodes and the lung leading to severe physiological consequences for the patient (reviewed in (Stahel and Weder, 2009)). Due to its severity, EPP is not suitable for every patient and they have to pass pivotal selection criteria.

The preferred systemic chemotherapy for mesothelioma treatment is the combination of pemetrexed and cisplatin, because it showed superiority concerning treatment response and median survival of the patients compared to a single agent used for treatment (Stahel and Weder, 2009). Another combined chemotherapy with rather palliative effect is the use of mitomycin C, vinblastine and cisplatin. Further, cisplatin was combined with carboplatin or gemcitabine, where no better efficacy was observed (reviewed by (Stahel and Weder, 2009)).

Multimodality therapy was initially performed with the idea to reduce local and distant relapses by combining chemo- or radiotherapy to surgery (Sugarbaker and Norberto,

1998). It includes neoadjuvant chemotherapy, a pre-treatment of the patient with therapeutic agents before starting the main treatment, together with EPP (reviewed by (Stahel and Weder, 2009)). This multimodality therapy increased median survival of MPM patients up to 23 months in Zurich and up to 19.2 months in the US (reviewed by (Stahel and Weder, 2009)).

To further improve MPM treatment research recently focuses on the identification of signaling pathways to develop targeted therapies. A signal pathway deregulated in cells leading to tumor growth and progression is the one induced by growth factors binding to receptor tyrosine kinases (TRK) (Belli et al., 2009), (van Meerbeeck et al., 2011). Under normal conditions the receptor tyrosine kinase pathway regulates cell proliferation, survival, cell movement and chemoresistance (Belli et al., 2009). TRK gets activated upon growth factor binding such as epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), insulin growth factor (IGF) or transforming growth factor β (TGF-β), where the latter binds to serine/threonine kinase receptors. It was observed that EGFR is overexpressed in MPM (reviewed by (Belli et al., 2009) and (van Meerbeeck et al., 2011)). Therefore gefitinib, an EGFR tyrosine kinase inhibitor, was investigated regarding its role in MPM treatment in a phase II clinical trial (reviewed by (Belli et al., 2009) and (van Meerbeeck et al., 2011)). Unfortunately no objective response was observed; therefore it was not further investigated (Belli et al., 2009), (van Meerbeeck et al., 2011). For a second EGFR inhibitor, erlotinib, evaluated in a phase II clinical trial, similar results were obtained (Belli et al., 2009), (van Meerbeeck et al., 2011). The observed un-responsiveness of EGFR tyrosine kinase inhibitors (EGFR-TKIs) might be explained by the absence of activating somatic mutation in EGFR-TKI domain which is strongly predictive of response to EGFR-TKIs as stated by reviewers (Belli et al., 2009), (van Meerbeeck et al., 2011). VEGF performing autocrine signaling during MPM development, was inhibited using a monoclonal antibody, bevacizumab (Belli et al., 2009), (van Meerbeeck et al., 2011). In a randomized phase II clinical trial bevacizumab was administered alone or in combination with cisplatin and gemcitabine (Belli et al., 2009), (van Meerbeeck et al., 2011). Also in this clinical trial no significant improvement in the outcome was observed (Belli et al., 2009), (van Meerbeeck et al., 2011).

3.2 THE CANCER STEM CELL THEORY

Somatic stem cells or tissue stem cells are cells with self-renewal potential and the capacity to differentiate into progenitor cells leading to the heterogeneity of cells within a tissue/organ (Reya et al., 2001). These characteristics are necessary to keep a tissue intact over a lifespan. Indeed, somatic stem cells are activated upon injury to repair lesions or are responsible for the renewal of the tissue by differentiation into fast proliferating progenitor cells, and to self-renew to keep the stem cell pool constant. Stem cells are a rare population within a tissue (Reya et al., 2001). Most stem cells are tissue specific, even though it could be observed that hematopoietic stem cells could differentiate into cells of non-hematopoietic tissues (Petersen et al., 1999). The best studied stem cells are the hematopoietic stem cells differentiating into all the cells of the blood system.

It seems that stem cell biology and tumor biology shares some aspects. Stem cells as well as tumor cells proliferate and differentiate to built-up/renew either an organ/tissue or a tumor bulk, respectively. Since stem cells are alive for a lifespan (due to their self-renewal capacity), the probability of accumulating mutations in stem cells leading to transformation into cancer (stem) cells (Reya et al., 2001) is increased. Due to this potential parallel between stem cell and tumor biology, a new model has been developed over the last decades to explain tumor growth. This so called cancer stem cell model (Figure 6B) claims that only few cells within a heterogeneous tumor are able to proliferate extensively and give rise to new tumors (Reya et al., 2001). The bulk tumor cells of this model have limited proliferation potential compared to the so called cancer stem cells (Reya et al., 2001). On the other hand the older, clonal evolution model (Figure 6A) claims that in a heterogeneous tumor each cell has the potential to proliferate extensively, but the probability of a single cell to proliferate extensively within a tumor is very low (Reya et al., 2001).

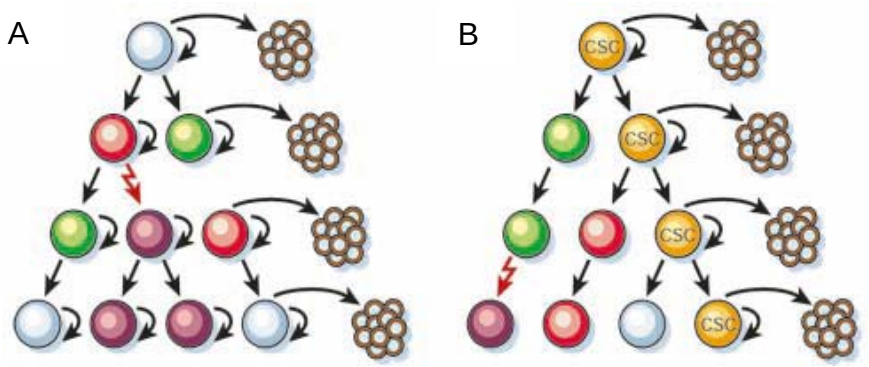

Figure 6: Two models explaining tumor growth. A) In the clonal evolution model each cell has the potential to proliferate extensively, but with only very low probability. B) In the cancer stem cell mode, only a minority of cells, the CSC proliferate extensively to built-up the whole tumor. Adapted from Reya et al. 2001.

The cancer stem cell model not only explains the growth of tumors, but also explains why tumors after their resection, chemotherapy and/or radiotherapy re-grow. Cancer treatment targets mainly bulk tumor cells leading to tumor shrinkage, but leaves cancer stem cells unharmed (Figure 7) (Reya et al., 2001). Additionally, it is speculated that due to drug transporter expression at the cell surface of cancer stem cells, they are more resistant against chemotherapeutics (Reya et al., 2001). The remaining viable cancer stem cells then lead to regrowth of the tumor.

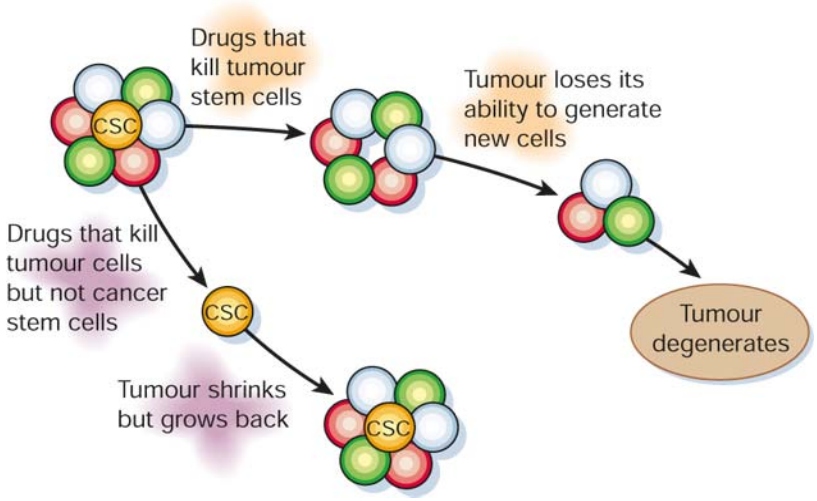

Figure 7: Targeting tumor bulk cells with chemotherapy shrinks the tumor, but it will regrow. Targeting cancer stem cells with chemotherapy will defeat the tumors ability of regrowth. Adapted from Reya, 2006.

3.2.1 LEUKEMIA INITIATING CELLS

Indications for the cancer stem cell model came from Lapidot et al. in 1994 (Lapidot et al., 1994). They suggested that in human acute myeloid leukemia (AML) a probable rare leukemia stem cell exists, because most AML cells are limited in their proliferative capacity (Lapidot et al., 1994). AML cells were sorted according to the expression of the cell surface marker CD34, usually expressed by pluripotent stem cells and progenitor cells of the bone marrow. After transplantation of $CD34^+$ and $CD34^-$ AML cells into SCID mice, only $CD34^+$ cells were able to engraft the mice (Lapidot et al., 1994). Then cells were sorted according to the expression of CD38 on $CD34^+$ cells. CD38 expressed by $CD34^+$ cells indicates their commitment for lineage. Therefore $CD34^+CD38^-$ AML cells specify an immature cell of the bone marrow (Lapidot et al., 1994). Also here, these $CD34^+CD38^-$ cells could engraft SCID and NOD/SCID (Bonnet and Dick, 1997) mice after transplantation whereas $CD34^+CD38^+$ cells were not able to do so (Bonnet and Dick, 1997), (Lapidot et al., 1994). Bonnet and Dick additionally showed that $CD34^+CD38^-$ cells in NOD/SCID mice reproduced the disease of the patient, indicating their capacity to differentiate (Bonnet and Dick, 1997). Furthermore they showed that these cells could self-renew in mice after re-engraftment of the donor's disease in second generation mice (Bonnet and Dick, 1997). All these findings indicate a hierarchical organization of AML (Bonnet and Dick, 1997) from where the cancer stem cell model developed.

Moreover, Costello et al. (Costello et al., 2000) showed in AML that the leukemia initiating cells $CD34^+CD38^-$ had a lower daunorubicin accumulation compared to $CD34^+CD38^+$ cells (Costello et al., 2000). Chemosensitivity of these cells against daunorubicin was tested and observed that $CD34^+CD38^-$ cells were higher proliferating after daunorubicin treatment compared to $CD34^+CD38^+$ cells (Costello et al., 2000). The increased proliferation rate accompanied by decreased apoptosis upon daunorubicin treatment and increased MRP expression, an ABC transporter well-known to efflux daunorubicin (Costello et al., 2000). Thereafter authors concluded $CD34^+CD38^-$ cells to be the immature leukemic progenitors with increased chemotherapeutic resistance.

3.2.2 CANCER STEM CELLS IN SOLID TUMORS

Almost 10 years after the identification of cancer stem cells in AML, Al-Hajj et al. (Al-Hajj et al., 2003) presented the first CSCs in a solid tumor. In their study, they inves-

tigated breast cancer for the presence of CSCs. For this purpose, authors considered the well-known heterogeneous expression of CD44 and CD24 (two adhesion molecules) to potentially differentiate tumorigenic from non-tumorigenic cells. By sorting breast cancer cells (from patients as well as passaged one to two times in mice) according to their expression of CD44, CD24 and Lineage cell surface markers, and after their injection into NOD/SCID mice, they identified $CD44^+CD24^-Lineage^-$ cells to be more tumorigenic *in vivo* compared to $CD44^+CD24^+Lineage^-$ cells (Al-Hajj et al., 2003). Additionally, they observed the self-renewal capacity of $CD44^+CD24^-Lineage^-$ cells *in vivo* giving rise to both tumorigenic $CD44^+CD24^-Lineage^-$ and non-tumorigenic $CD44^+CD24^+Lineage^-$ cells in second generation mice (Al-Hajj et al., 2003) recapitulating the heterogeneity of the tumor they derived from. This observation could not be made for the $CD44^+CD24^+Lineage^-$ cells. Finally authors claimed that breast cancer is also hierarchically organized, because early multipotent epithelial progenitor cells also do express CD44 (Al-Hajj et al., 2003).

In the same year, Singh et al. identified in 4 phenotypic different brain tumors the brain tumor stem cells which were in an undifferentiated state, not expressing differentiation markers (β-tubulin 3 for neurons and GFAP for astrocytes) (Singh et al., 2003). Nevertheless these brain tumor stem cells were characterized by the expression of the neural stem cell surface marker CD133 (prominin-1). $CD133^+$ brain tumor cells exhibited increased self-renewal as well as proliferating capacity compared to $CD133^-$ brain tumor cells in a sphere formatting assay (Singh et al., 2003). Furthermore, $CD133^+$ cells recapitulated the tumor they derived from (Singh et al., 2003). Unfortunately it later became clear that CD133 as a sole marker to identify CSCs in glioma is not enough (Campos and Herold-Mende, 2011), because it was observed that CD133 positive as well as negative cells induced tumor growth *in vivo* (Shmelkov et al., 2008).

From then on in many solid tumors cancer stem cells were identified according to corresponding tissue stem cell markers, reviewed in Visvader & Lindeman (Visvader and Lindeman, 2008) and Cho & Clarke (Cho and Clarke, 2008).

3.2.3 Functional Assays to Identify Cancer Stem Cells

The cancer stem cell model claims that mutated somatic stem cells are the source of tumor growth/relapse (see section 3.2). Hence, it is possible to use known stem cell surface markers to isolate putative cancer stem cells and investigate their capaci-

ty/potency of tumor initiation, self-renewal and differentiation. In tissues, in which no stem cell surface markers have been identified to date, such an approach is not possible. Therefore, another approach would be to investigate functional markers of stem cells which are kept by transformed cancer stem cells as suggested by Ginestier et al. (Ginestier et al., 2007). To date, two functional assays developed to identify somatic and cancer stem cells: the aldehyde dehydrogenase assay (section 3.2.3.1) and the side population assay (3.2.3.2).

3.2.3.1 THE ALDEHYDE DEHYDROGENASE ASSAY

Aldehyde dehydrogenase 1 is a cytosolic enzyme expressed by the *ALDH1* gene. This enzyme oxidizes intracellular aldehydes and therewith detoxifies aldehydes (Ginestier et al., 2007). ALDH1 was observed to be highly expressed in hematopoietic stem cells most probably to protect the cells from toxic aldehydes.

ALDH positive cells can be isolated using flow cytometer technics. Therefore cells are probed with a fluorescent aldehyde substrate (BAAA, BODIPY – aminoacetaldehyde) which diffuses across the cell membrane of living cells (Storms et al., 1999), (Storms et al., 2005). The substrate gets oxidized by aldehyde leading to a fluorescent product staying within the cell (negatively charged BAA^-, BODIPY – aminoacetate). It then can be excited at 488 nm and the emission of which can be detected with a 530/30 band pass filter in a flow cytometer and/or sorted by FACS (StemCell Technologies), (Storms et al., 1999), (Storms et al., 2005). Data are analyzed in a dotplot (SSC vs. BODIPY) and cells would be sorted according to their BAAA signal with potential stem cells isolated from a $SSC^{lo}ALDH^{br}$ gate (SSC low and ALDH bright; Figure 8), because cells with high aldehyde activity, the potential cancer/tissue stem cells, stain brighter than cells with less activity.

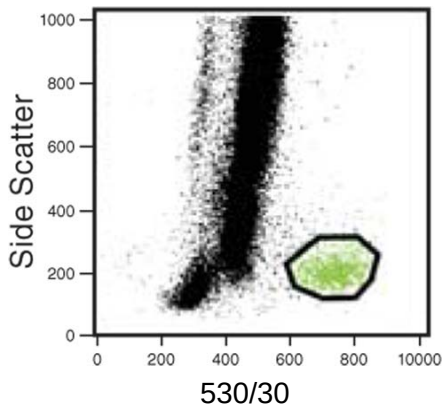

Figure 8: Representative flow cytometery analysis using the aldehyde dehydrogenase assay. Green: $SSC^{lo}ALDH^{br}$ cells. Adapted from StemCell Technologies.

3.2.3.2 THE SIDE POPULATION ASSAY

Another functional assay, called side population assay was developed by Goodell et al. in 1996 (Goodell et al., 1996). By using the DNA staining dye Hoechst 33342 to mark living cells of the whole murine bone marrow, they identified a so called side population (SP) after exciting Hoechst 33342 at 350 nm (UV light) and detection of its emission at two different wavelengths: Hoechst red (675 nm long pass filter) and Hoechst blue (450/20 nm band pass filter) (Goodell et al., 1996). This detection strategy of Hoechst leads to a better separation of its signal. This is of need, because Hoechst has a very broad emission spectrum ranging from 400 – 600 nm (Watson et al., 1985). A second DNA dye in use to detect SP cells is DyeCycleViolet (Mathew et al., 2009), (She et al., 2008), (Telford et al., 2007). It shares emission and cell permeability characteristics with Hoechst 33342 (reviewed by (Telford et al., 2007)). The advantage of DCV is its longer excitation wavelength (369 nm, (Telford et al., 2007)). Exciting DCV at 405 nm reveals emission of 20%, whereas Hoechst excited at 405 nm reveals only 5% emission (Telford et al., 2007). This is of importance, because most flow cytometers and FACS machines are only equipped with a 405 nm laser, not with a UV laser actually needed to excite Hoechst and DCV best.

Goodell et al. characterized the SP cells of whole murine bone marrow according to their expression of cell surface markers as Sca-1^{+}Lin$^{neg/low}$ cells, representing the murine HSCs (Goodell et al., 1996). Additionally these SP cells were enriched for stem cell activity *in vivo* and contributed to both lymphoid and myeloid lineages *in vivo* (Goodell et al., 1996).

This functional assay is based on the expression of drug transporters in the cell membrane of cancer/tissue stem cells. The drug transporters efflux substrates like DNA staining dyes. Therefore, cells are stained with Hoechst 33342 or DCV in the presence or absence of a drug transporter inhibitor. The side population is defined in the lower left corner of the dotplot analysis (Hoechst/DCV red vs. blue), representing cells which efflux the DNA dyes, so-called Hoechst/DCV low cells. By comparing dotplots of minus inhibitor treated samples to plus inhibitor treated samples, the side population is precisely defined as the population disappearing in inhibitor treated samples (Supplementary Figure 1 and Figure 9).

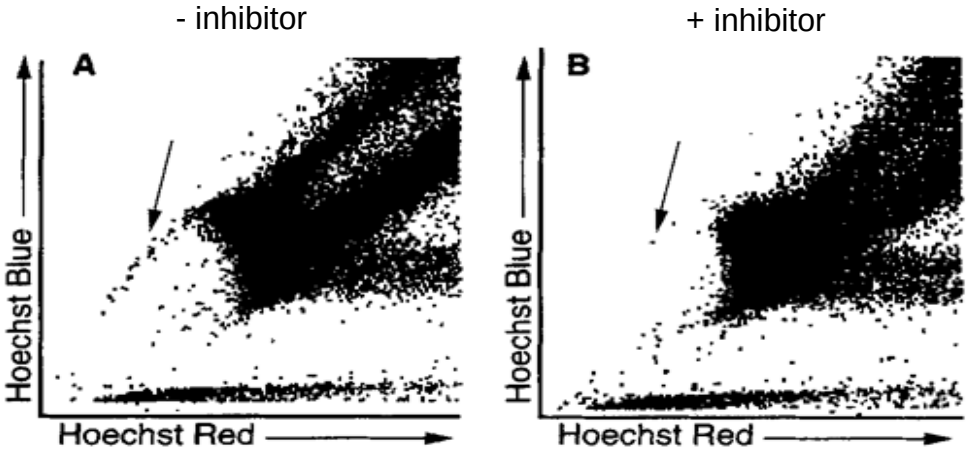

Figure 9: Flow cytometry dotplot analysis. Side population is indicated with the arrow (A), which disappears in the presence of the drug transporter inhibitor (B). Adapted from Goodell 1996.

3.2.4 ABCG2 Expressing Side Population Cells in Normal Tissue

Already Goodell et al. stated that the responsible factor for the side population phenotype observed might be a drug transporter like MDR/p-glycoprotein belonging (like MRP) to the ABC drug transporter family and which efflux Hoechst 33342 leading to the Hoechst 33342 low (i.e. SP) profile in a flow cytometry analysis (Figure 9) (Goodell et al., 1996). To shed light on the transporters expressed in SP cells, Zhou et al. investigated the SP in bone marrow cells of MDR1 knock-down mice and observed that knocking down MDR1 does not abolish the SP fraction (Zhou et al., 2001) indicating that MDR1 is not the ABC transporter responsible for the SP phenotype. Nevertheless a different ABC transporter was responsible for this phenotype since ATP depletion as well as the incubation with a functional inhibitor of several ABC transporters diminished the SP fraction in these MDR1 knock-down mice (Zhou et al., 2001). In their study ABCG2, another ABC transporter, was identified to be highly expressed in SP cells as well as in $CD34^-$ murine bone marrow stem cells (including hematopoietic, mesenchymal and endothelial cells) (Zhou et al., 2001). In addition, higher expression of ABCG2 was observed in SP cells of rhesus monkey bone marrow and mouse skeletal cells compared to non-SP (NSP) cells (Zhou et al., 2001). Furthermore, overexpression of ABCG2 in hematopoietic stem cells inhibited their differentiation (Zhou et al., 2001). Scharenberg et al. (Scharenberg et al., 2002) also identified ABCG2 to be the drug transporter responsible for the side population phe-

notype in hematopoietic stem cells. This was confirmed in a later study of Zhou et al. (Zhou et al., 2002) where they correlated $K^+S^+L^-$ hematopoietic stem cells with ABCG2 expressing SP cells with *in vivo* repopulating capacities.

SP cells harboring putative tissue stem cells were identified for multiple different tissues like in skeletal muscle (Montanaro et al., 2004), mammary glands (Welm et al., 2002), testis (Lassalle et al., 2004) and limbal[7] epithelium (Watanabe et al., 2004).

3.2.5 ABCG2 Expressing Side Population Cells in Cancers

Beside the fact that murine bone marrow includes a SP fraction with stem cell activity and potency (Goodell et al., 1996) and $CD34^+CD38^-$ AML initiating cells do so as well (Bonnet and Dick, 1997), (Lapidot et al., 1994), Wulf et al. (Wulf et al., 2001) next detected in the bone marrow and the peripheral blood of acute myeloid leukemia patients SP fractions with leukemia initiating potential. Later on, SP fractions with increased *in vivo* tumorigenic potential were detected in cell cultures or tissues derived from different solid tumors. Kondo et al. (Kondo et al., 2004) isolated from C6 glioma cell lines SP cells with characteristics of multipotent cancer stem cells *in vivo*. Sorted SP cells had increased tumor growth potency compared to NSP cells *in vivo* and tumors developed from C6 glioma SP cells expressed neuron and glia cell markers suggesting the differentiation capacity of SP cells *in vivo* (Kondo et al., 2004). Further, Hirschmann-Jax et al. identified in neuroblastoma samples SP cells with increased *in vitro* proliferation and self-renewal potential (Hirschmann-Jax et al., 2004).

In the meanwhile SP cells with cancer stem cell/tumor initiating characteristics were identified in ovarian cancer (Szotek et al., 2006), lung cancer (Ho et al., 2007), (Loebinger et al., 2008), hepatocellular carcinoma (Chiba et al., 2006), nasopharyngeal carcinoma (Wang et al., 2007), bone sarcomas (Murase et al., 2009), pancreatic carcinoma (Wang et al., 2009) and in glioblastoma (Fukaya et al., 2010).

3.3 The ABCG2 Drug Transporter

3.3.1 ATP-Binding Cassette Transporters

The ABCG2 drug transporter responsible for the SP phenotype is a member of the ATP-binding cassette transporter family, the biggest family of active transporters (49

[7] Border of the cornea (transparent front part of the eye covering the iris and pupil) and the white of the eye

members identified so far, (Matsson et al., 2011)). In eukaryotic cells they are always efflux pumps, exporting substrates across the cell membrane and against a concentration gradient. Therefore in almost all cases it is an energy dependent step (Scharenberg et al., 2002) using ATP-binding and its hydrolysis as source of energy (Robey et al., 2009). Beside the role of ABCG2 in the SP phenotype, ABCG2 together with the multidrug resistance protein, p-glycoprotein ABCB1 (P-gp/MDR-1) and the multidrug resistance-associated protein-1 ABCC1 (MRP-1) (Robey et al., 2009), (Scharenberg et al., 2002), they are frequently associated with the multidrug resistance phenotype observed in cancer cells. The overexpression of these drug transporters protects cancer cells against chemotherapeutics via increased drug efflux decreasing intracellular drug concentrations (Robey et al., 2009).

Alterations in ABC drug transporter expression, most probably due to mutations, are not only observed in cancers leading to chemoresistance, but also in many other diseases: ABCG5 and/or ABCG8 are mutated in sitosterolemia[8] or ABCA1 is mutated in high-density lipoprotein deficiency (Tangier disease) (Matsson et al., 2011), (Velamakanni et al., 2007).

ABC drug transporters in general have a structure including a pair of transmembrane (TM) domains which are hydrophobic and give most of its specificity to the transporter. Further, they include a set of two ATP-binding domains (or nucleotide-binding folds, NBFs) (Robey et al., 2009) according to their sequence, the transporters are grouped into subfamilies (A-G in the human genome). While most genes encode full transporters, the G subfamily encodes only half-transporters which have to dimerize or maybe also oligomerize as homo- or heterodimers to acquire its full activity (see Oligomerization section 3.3.2.4) (Robey et al., 2009).

3.3.2 ABCG2

Initially, ABCG2 was identified as a drug transporter responsible for the multidrug resistance observed in cancer cells. Before the association of ABCG2 with the multidrug resistance phenotype of cancer cells, Pgp and MRP were known drug transporters responsible for this phenotype. Nevertheless, Doyle et al. investigated the multidrug resistant breast cancer cell line MCF-7/AdrVp, which was known not to overexpress Pgp or MRP (Doyle et al., 1998). By RNA fingerprinting Doyle et al. identified a sequence encoding for what they called breast cancer resistant protein

[8] Rare autosomal recessively inherited lipid metabolic disorder

(BCRP), which was overexpressed in the chemoresistant breast cancer cell line MCF-7/AdrVp, but not in its parental cell line MCF-7 or in its partially reverted MCF-7/AdrVpPR subline (Doyle et al., 1998).

3.3.2.1 ABCG2 EXPRESSION IN DIFFERENT TISSUES

The ABCG2 drug transporter is expressed in many different organs like in the placenta (Allikmets et al., 1998), brain, brain-blood barrier, prostate, liver, testis, ovary, small intestine and human embryonic stem cells (Apati et al., 2008) at different levels (reviewed by (Robey et al., 2007, Robey et al., 2009)).

In human embryonic stem cells it could be shown that upon differentiation the cells lost ABCG2 expression (Apati et al., 2008).

3.3.2.2 REGULATION OF ABCG2 EXPRESSION AND FUNCTION

ABCG2 is expressed by the human *ABCG2* gene located on chromosome 4q22 expanding over 66 kb including 16 exons and 15 introns (Robey et al., 2007), (Robey et al., 2009). The translational start codon resides in exon 2. Exon 3 harbors the Walker A site. Its expression might be regulated at promoter level by sex hormones (Robey et al., 2007), its methylation status (Robey et al., 2007) and by hypoxia (Martin et al., 2008). Indeed, under hypoxia, HIF-2α leads to increased ABCG2 expression, protecting the cells from oxidative stress (Martin et al., 2008) via increased efflux of its known substrate porphyrin which is induced upon hypoxia and which causes mitochondrial death (reviewed by (Krishnamurthy et al., 2004)).
Mutation at Arg^{383} alters glycosylation of ABCG2 and retains it in the endoplasmic reticulum leading to proteasomal degradation and decreased ABCG2 protein level (Ni et al., 2010). Furthermore, some single nucleotide polymorphisms (SNPs) can reduce ABCG2 membrane expression decreasing efflux efficiency (Robey et al., 2007).

Further, ABCG2 can be regulated at a functional level by inhibiting its insertion into the cell membrane, which is needed to fulfill its transport activity. Bleau et al. observed that inhibition of the PI3K/Akt pathway resulted in decreased SP fraction in gliomas and ABCG2 translocated from the cell membrane into the cytoplasm (Bleau et al., 2009). Mogi et al. observed the same for bone marrow cells, where ABCG2 expressed by sorted SP cells translocated from the cell membrane into the cytoplasm after PI3K/Akt pathway inhibition compared to untreated SP cells (Mogi et al., 2003).

An additional post-translational regulation of ABCG2 protein level and thus function is the formation of disulphide bonds in the extracellular loop of the protein structure (see below section 3.3.2.3). The formation of disulphide bonds is necessary for the stability of ABCG2 (Wakabayashi et al., 2007). Disulphide bonds are formed in an oxidative environment, which is found in the extracellular space. If the pH of incubation media drops (acidic environment), oxidative situation changes to a reductive environment and by losing the disulphide bond, ABCG2 proteins get instable and thus degraded in an ubiquitin/proteasome dependent manner (Wakabayashi et al., 2007).

3.3.2.3 PROTEIN STRUCTURE

Since 1990 researchers are investigating the structure of ABC transporters. The different domains (ATP-binding domain, nucleotide-binding domain etc.) were already known by then, but only for about 30 by then identified ABC transporters (Hyde et al., 1990). Additionally, they already observed substrate specificity of different subfamilies of the ABC transporters (Hyde et al., 1990).

ABCG2 is built up by 665 amino acids leading to a protein of 72 kDa in size (Robey et al., 2009), (Velamakanni et al., 2007). Its transmembrane (TM) domain is located at its C-terminus (residues 361 – 655) and its ATP-binding domain (NBD) at its N-terminus (Figure 10B). The TM domain provides the transporter's substrate specificity (Velamakanni et al., 2007). The NBDs include three conserved sequences: Walker A and B motifs and the ABC signature motif giving its hallmark to each individual ABC transporter (Velamakanni et al., 2007). The transmembrane domain possibly harbors six TM (α-helices) segments forming the pathway for the substrates to cross the lipid bilayer (Higgins and Linton, 2004). Between transmembrane segments five and six there is an extracellular loop (Robey et al., 2009) and it seems that ABCG2 includes three N-linked glycosylation sites (Robey et al., 2009). Alterations in specific residues of the extracellular loop may influence the oligomerization of the ABCG2 half-transporter, its localization and therewith its function (Robey et al., 2009).

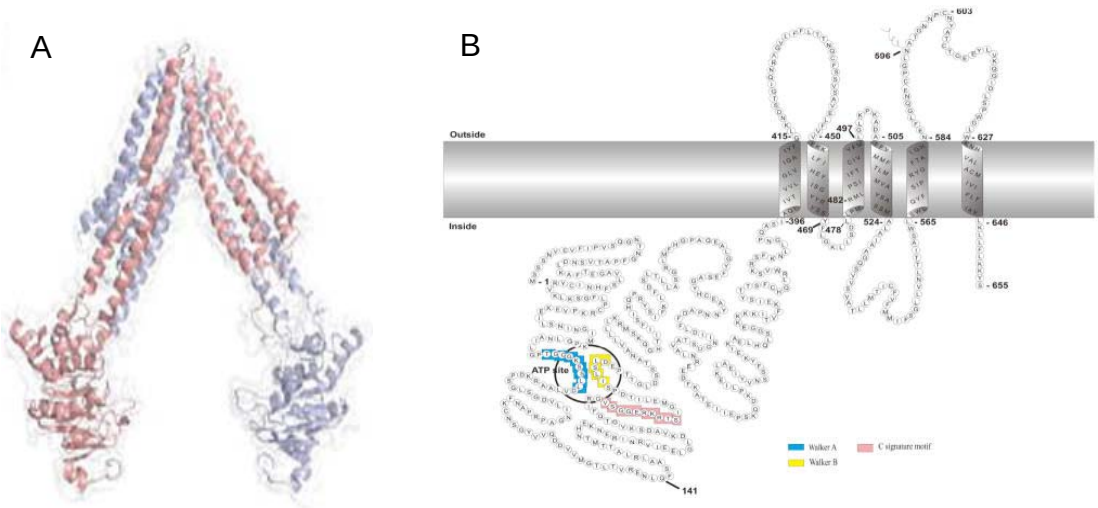

Figure 10: ABCG2 schematic model (A). ABCG2 membrane topology (B). Adapted from Ni et al., 2010.

3.3.2.4 OLIGOMERIZATION

ABCG2 is expressed as a half-transporter, which has to oligomerize to get its full activity. To investigate the oligomeric state of ABCG2, Xu et al. used a mild ionic detergent leaving non-covalent interactions of protein subunits intact. With this method they observed that ABCG2 most likely occurs as homotetramer in living cells (Xu et al., 2004) not as homodimer as previously thought. Nevertheless, it is not known if the most abundant homotetramer is also the active form of ABCG2 or if it serves as a regulator for the level of functional ABCG2 where the homodimer would represent the active ABCG2 form (Xu et al., 2004). This means, if one oligomer forms, it decreases the level of the other and therewith decreases the active ABCG2 form in the cell membrane (Xu et al., 2004). McDevitt et al. investigated the 3D structure of ABCG2 using electron microscopy and also claims that ABCG2 forms a tetramer of dimers (McDevitt et al., 2006).

3.3.2.5 ATP HYDROLYSIS AND ITS EFFECT

On the basis of substrate translocation across the cell membrane via ABC transporter is the ATP-switch model (reviewed by (Higgins and Linton, 2004) and illustrated in Figure 11). The initial step of the transport cycle is performed by substrate binding to its high-affinity site on the TM domain followed by increased affinity of NBDs for ATP (Higgins and Linton, 2004). This lowers the activation energy for ATP-dependent dimerization (Higgins and Linton, 2004). Via binding of two ATPs to the NBDs, they undergo a conformational change resulting in the closed dimer (Higgins and Linton,

2004). This represents the power stroke upon which the TM domain undergoes a conformational change releasing its bound substrate into the extracellular space by reducing its binding affinity (Higgins and Linton, 2004). Next the ATPs get hydrolyzed leading to an intermediate transition-state in which the NBD closed dimer gets destabilized (Higgins and Linton, 2004). This initiates the reset of the transporter into its basal configuration (Higgins and Linton, 2004). Therefore P_i and ADP are released successively restoring the transporters configuration to be ready for the next cycle (Higgins and Linton, 2004).

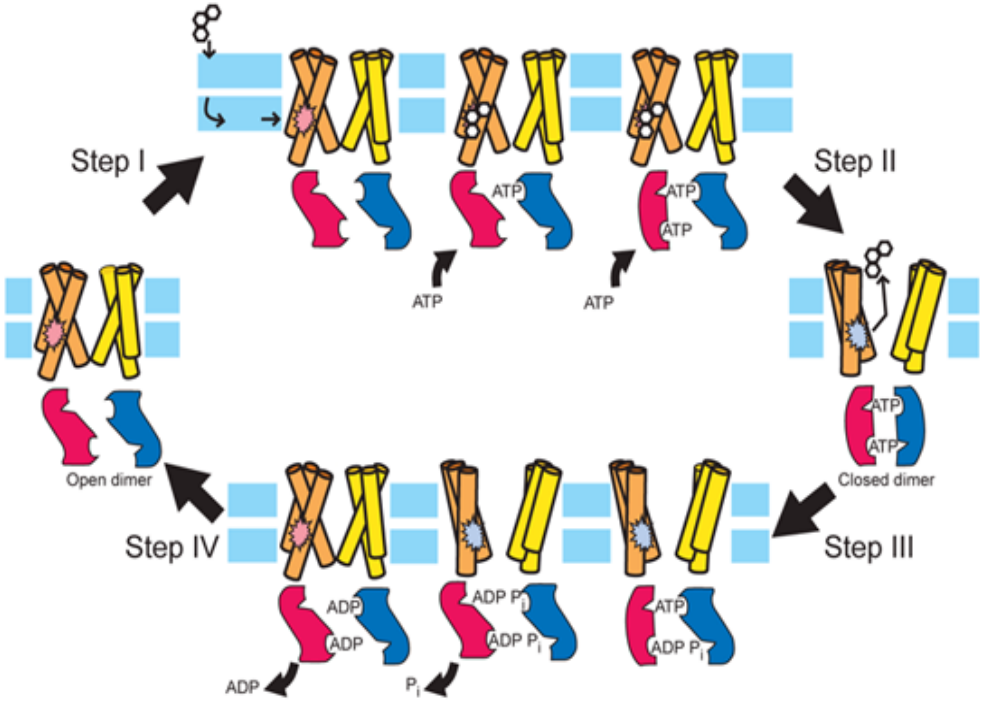

Figure 11: The ATP-switch model. Adapted from Higgins and Linton, 2004.

3.3.2.6 ABCG2 Substrates and Inhibitors

The association of ABCG2 to multidrug resistance in cancer cells led to the identification of many ABCG2-specific chemotherapeutics. Well known chemotherapeutics effluxed via ABCG2 are, within others: mitoxantrone, topotecan, methotrexate, daunorubicin, doxorubicin, gefitinib and imatinib (reviewed by (Ni et al., 2010), (Robey et al., 2007)). Additionally, due to its expression in organs like the placenta or brain-blood barrier, it also effluxes non-chemotherapeutics like carcinogens (aflatoxin B1), antivirals, antibiotics and others like flavonoids (Vitamin P), riboflavin (vitamin

B_2/E101) and vitamin K3 (reviewed by (Ni et al., 2010), (Robey et al., 2009)). Even though these substrates are well known to be transported by ABCG2, there is a remarkable overlap of the substrate specificity between ABCG2 and MDR1. Nevertheless, MDR1 generally transports hydrophobic substrates whereas ABCG2 transports both hydrophobic as well as hydrophilic substrates (Ni et al., 2010).

The first very specific ABCG2 inhibitor reported was fumitremorgin C (FTC) (Rabindran et al., 2000). Nevertheless, other inhibitors were reported earlier but these were also inhibiting other ABC transporters like MDR1 and MRP1 (Robey et al., 2009).

Even though the list of substrates and inhibitors is large, no structure-function relationship could be identified so fare which could explain the requirements to be a substrate or inhibitor for ABCG2 (Robey et al., 2009).

4 AIM OF THE THESIS

As summarized above, patients diagnosed with malignant pleural mesothelioma (MPM) have a poor prognosis. Treatment is mostly palliative and patients mostly die within one year after diagnosis. Where chemotherapy is applied, patients develop resistance against chemotherapeutics. Therefore it is of great importance to improve mesothelioma treatment.

For this purpose and according to the cancer stem cell model, the aim of this thesis was to identify cancer stem cells in malignant pleural mesothelioma and to characterize them in order to gain information about tumor recurrence after resection and the development of chemoresistance.

Due to the lack of known mesothelial stem cells and its markers we used a more universal approach for the identification of potential cancer stem cells in malignant pleural mesothelioma. The side population assay is based on functional ABCG2 drug transporter expressed by cancer stem cells and able to efflux DNA dyes such as Hoechst 33342 or DCV. This assay was used to isolate SP cells of MPM which were then tested for their tumorigenic potential in NOD/SCID mice and further characterized concerning their self-renewal capacity, stem-like cell properties and their possible mesenchymal origin. In addition SP derived cells were tested for their chemoresistance.

5 RESULTS

5.1 MANUSCRIPT: PLEURAL MESOTHELIOMA SIDE POPULATIONS HAVE A PRECURSOR PHENOTYPE AND ARE SIMILAR TO PATIENT'S RELAPSES

Claudia Frei, Isabelle Opitz, Alex Soltermann, Bruno Fischer, Ubiratan Moura, Hubert Rehrauer, Walter Weder, Rolf Stahel and Emanuela Felley-Bosco

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Authors' contributions:

CF and EFB contributed to conception and design;
CF, IO, UM, BF and EFB contributed to the acquisition of data;
CF, EFB, AS and HR contributed to the analysis and interpretation of data;
CF, EFB, IO, AS, HR have been involved in drafting the manuscript or revising it critically for important intellectual content;
WW and RS have given final approval of the version to be published.

Pleural mesothelioma side populations have a precursor phenotype and are similar to patient's relapses

Running title: mesothelioma epithelial-mesenchymal transition

Claudia Frei[1], Isabelle Opitz[2], Alex Soltermann[3], Bruno Fischer[1], Ubiratan Moura[1], Hubert Rehrauer[4], Walter Weder[2], Rolf Stahel[1] and Emanuela Felley-Bosco[1]

[1] Molecular Oncology, Clinic of Oncology, University Hospital Zürich, Switzerland

[2] Division of Thoracic Surgery, University Hospital Zürich, Switzerland

[3] Institute of Surgical Pathology, University Hospital Zürich 8091 Zürich, Switzerland

[4] Functional Genomic Center Zurich, 8057 Zurich, Switzerland

Corresponding author: Emanuela Felley-Bosco
Laboratory of Molecular Oncology
Clinic for Oncology
University Hospital Zurich
Haeldeliweg 4
8044 Zurich, Switzerland
Phone: +41 44 6342878
Fax: +41 44 6342872
Email: emanuela.felley-bosco@usz.ch

Abstract

DyeCycleViolet was used to set up the side population (SP) functional assay aimed at identifying subpopulations of malignant pleural mesothelioma (MPM) tumor cells with chemoresistance phenotype associated with ABCG2 transporter activity. Self-renewal, chemoresistance and tumorigenicity were tested for SP and non-SP (NSP) cells. Tumors were characterized by mesothelin, calretinin, N-cadherin, D2-40 and Wilms tumor 1 (WT1) immunohistochemistry. Surface expression of mesenchymal stem cell markers CD90, CD73 and CD105 was investigated in SP and NSP cells. We identified SP cells with self-renewal properties and increased chemoresistance in MPM cell lines and tumor derived primary cell cultures. Compared to the non-SP fraction (NSP), the SP fraction led to the development of tumors including cells with mesothelium precursor phenotype characterised by mesenchymal morphology, being WT1 negative but cytoplasmic D2-40 positive, and having a tendency of increased tumorigenicity. The same phenotypic shift was observed in patients with relapsing tumors after chemotherapy. Furthermore the SP cells were enriched in CD105 $^{-/low}$ expressing cells which were small sized and had increased tumorigenicity compared to CD105 ^{high} cells. Taken together our results support the hypothesis that MPM CD105$^{-/low}$, chemoresistant, small sized SP cells may constitute the cellular pool out of which recurrence develops. Further characterization of mechanisms of chemoresistance and self-renewal should lead to targets specific for this subpopulation in MPM patients.

Introduction

Asbestos-related malignant pleural mesothelioma (MPM) is expected to peak in 2020 [1]. Survival rate are still around one year (depending on the age and treatment) after diagnosis [2]. Objective chemotherapy response is achieved in less than 50% of patients and has limited duration [3].

In order to gather a better understanding of MPM biology which may ultimately help defining better therapeutic strategies we considered parallel aspects between MPM development and other tumors linked to chronic injury [4]. The asbestos fibres-induced MPM development is due to accumulation of inhaled asbestos fibres in the pleural space and subsequent damage of the mesothelium [5]. Chronic tissue repair follows, activating stem cell signalling pathways to regenerate the tissue but, because of persistent system stimulation, oncogenic events occur leading to tumor formation (reviewed in [6]). The presence within the latter of a population of cancer stem cells (CSC) highly resistant to chemotherapy might be the cause of tumor recurrence. Hence, the aim of our study was originally to identify and characterize MPM CSC. The latter, which have the capability to create exact tumor phenocopies when transplanted from one mice to the next, have been proposed to exist [7] based on the pioneer work identifying leukemic stem cells as $CD34^{+}/CD38^{-}$ cells, like hematopoietic stem cells (HSC), in human acute myeloid leukemia (AML) [8], [9]. The presence of CSCs in solid tumors was identified for the first time as a small subset of heterogeneous breast cancer cells which were phenotypically distinct and characterized as $CD44^{+}/CD24^{-/low}$ [10]. Since then, for many solid tumors cancer stem cells have been identified using corresponding tissue stem cell surface markers (reviewed in [11]). Normal mesothelium stem cell surface markers are not yet available to identify potential MPM CSCs. Hence, we used a functional assay which identifies a small and distinct subset of cells, called “side population” (SP), with phenotypic markers of multipotential HSC after staining bone marrow with the DNA staining dye Hoechst 33342 [12]. The SP is due to the expression of functional ATP-binding cassette (ABC) transporters [13]. When living cells are stained with Hoechst 33342, SP cells do efflux the DNA staining dye via their ABC transporters. When cells are co-incubated with ABC transporter inhibitors verapamil or fumitremorgin C (FTC), Hoechst 33342 is no longer effluxed leading to a shift in the dual emission wavelength fluorescence activated cell sorting (FACS) analysis upon which the SP

can be identified. The ABCG2 drug transporter is responsible for the SP in the bone marrow [13], [14]. Although this assay has already been applied to mesothelioma cell lines [15] several issues are still needed to be addressed. First, self-renewal of sorted SP has not been investigated yet, second tumorigeniciy has been determined only for SP and NSP cells sorted from MS-1 cell line, which has a biphasic histotype and no differences were found. In our study, those questions were addressed using primary cultures from xenografts, either from a cell line or from a patient derived tumor instead of cell lines. In addition, surface phenotype and chemoresistance were characterized.

Material and Methods

Tissue samples

Human tumor specimens were obtained and processed as previously described [16], [17].

Cell lines and primary cell cultures

The human promyelocytic leukemia cell line HL60/Dox selected by chronic exposure to doxorubicin was kindly provided by Dr. M. Andreev (Department of Blood and Marrow Transplantation, UT MD Anderson Cancer Center, Huston, Texas) and maintained in RPMI 1640 (Sigma – Aldrich) supplemented with 10% FCS (Omnilab), 1% penicillin/streptomycin and 1mM L-Glutamine (both obtained from GIBCO). The breast cancer cell line MCF-7 was maintained in RPMI 1640 supplemented with 10% FCS, 1% penicillin/streptomycin and 2mM L-Glutamine. Primary MPM cell cultures were established from surgical specimens or xenografts as follows: tumors were blended into small pieces of 1 to 3 mm^2, then fragments were directly added to culture medium adapted from Connell et al. [18] (DMEM/F12+GlutaMax (GIBCO), 15% FCS, 0.4 μg/ml hydrocortisone (Sigma – Aldrich), 10 ng/ml EGF (epidermal growth factor, GIBCO), 1% ITS (insuline, transferrin, selenium, GIBCO), 1 mM sodium pyruvate (Sigma – Aldrich), 100 μM beta-mercaptoethanol (Fluka), supplemented with 1% non-essential amino acids (GIBCO) and 30% conditioned medium), or were incubated with collagenase 3 (200 U/ml, Worthington Biochemical

Corporation, New Jersey, USA) and hyaluronidase (100 U/ml) for 6h at 37 C followed by digestion with 0.25% Trypsin/EDTA (ethylenediaminetetraacetic acid, GIBCO) for 2 min at 37°C and with 20 mg/ml DNAseI (Worthington Biochemical Corporation, New Jersey, USA) for additional 2 min at 37°C. At the end of the collagenase digestion, tissues were filtered through a 70 µm cell sieve. The filtrate was centrifuged and the pellet resuspended in culture medium. The mesothelioma cell lines ZL34 and ZL55 were established in our laboratory [19]. The MPM cell line H28 and Met5A, a mesothelial cell line obtained by SV40 transformation of mesothelial cells [20], were obtained from ATCC. All cell lines used in this study were authenticated by DNA fingerprinting (Microsynth, Switzerland).

Gene expression analysis

Selected gene expression analysis was performed as previously described [16], [17] . Additional primers are listed in the Supplementary Table I. The heatmap was produced with R using default options on ΔCt raw data. We used the logarithmic expression values and subtracted for each gene its mean expression.

Measurement of cell growth

To test sensitivity to mitoxantrone, cells were first incubated for 1 h with mitoxantrone (Sigma – Aldrich) (1 ng/ml) then verapamil (20 µM) was added or not for 1 h. Both drugs were suspended in medium without serum. After incubation, drugs were removed and cells were grown for further 3 days in serum containing medium. Sensitivity to cisplatin (0 – 16 µM) was tested in MPM medium containing 0.5% FCS. Cell growth was determined as previously described [21].

Western blot analysis

Western blotting was done as described [16]. The Western blots are representatives of 2 – 5 independent experiments.

Side population analysis

Cells were stained with DCV using a previously described method [22], [23], [24] and adapted as follows. Briefly, cells were washed with PBS, trypsinized and resuspended in DMEM:F12 supplemented with 2% FCS, 10 mM 4-(2-hydroxyethyl)-

1-piperazineethanesulfonic acid (HEPES, Sigma – Aldrich), 0.4 μg/ml hydrocortisone, 10 ng/ml EGF, 1% ITS, 100 μM β-mercaptoethanol, 1 mM sodium pyruvate, 2 mM L-Glutamin and 1% non-essential amino acids. As positive controls, HL60/Dox cells were used. Cells were incubated with DCV (0.5 μM, Invitrogen) in the absence or presence of verapamil (50 μM; Sigma – Aldrich) at 37°C for 90 min in the dark mixing every 15 min. Then cells were spun down at 4°C and resuspended in ice-cold Hanks' balanced salt solution (HBSS, GIBCO) supplemented with 10 mM HEPES and 5 mM EDTA (Amresco). Viable cells were gated with propidium iodide (PI, 2 μg/ml). Cells were gated on the FACSAria (Becton Dickinson) FSC and SSC, a live gate was placed on cells excluding PI (Ex 488 nm, Em 610/20 nm) and single cells were gated based on FSC (FSC lin vs pulse width) and SSC (SSC lin vs pulse width). SP was determined by a gate placed on 450/40 vs 530/30 nm emission dot plot on a logarithmic amplified fluorescence scale after DCV excitation at 407 nm [25]. The SP gate was identified using verapamil which blocks drug efflux. These settings gave the same results as 450/40 vs 650 nm longpass and allowed a direct comparison with results on surface phenotyping using the CyAn ADP analyzer (Beckman Coulter), described below where only the 530/40 nm filter for 405 nm excitation was available. After sorting cells were cultured or processed for mice xenografts.

Immunohistochemistry

De-paraffinized sections were subjected to antigen retrieval using Tris/EDTA (1 mM/0.1 mM, pH9) or pepsin digestion (for WT1). Following quenching in 0.3% H_2O_2 (20 min) and permeabilization in 0.05% Saponin (Fluka, 5 min), blocking was performed in 2% bovine serum albumin (BSA) in PBS with 1% horse serum (Vector Laboratories, 20 min) at room temperature. Sections were incubated with primary antibodies (in supplementary figures: N-cadherin 6G11, 1:50; DAKOCytomation, mesothelin 5B2, 1:30; NovoCastra™ Laboratories, calretinin 1:50, Abcam) overnight at 4°C. Negative controls were incubated with secondary biotinylated antibody only (Vectastain® Elite® ABC Kit, Vector Laboratories). Sections were washed with PBS and incubated with secondary biotinylated antibody for 45 min at room temperature. Staining was visualized using 3,3'-diaminobenzidine tetrahydrochloride (Sigma – Aldrich), counterstained with Vector® Hematoxilyn QS (Vector Laboratories) and analysed either using a Leica DM IRBE microscope and image acquisition with Nikon

Coolpix and Retiga Cooled Color 12-bit camera and QCapture (QImaging) or using a Zeiss Mirax Midi Slide Scanner and image acquisition with a 3CCD colour camera and Mirax Viewer (Zeiss). A minimum of 200 cells was evaluated.

Surface phenotyping

Cells resuspended in ice-cold PBS/2mM EDTA were incubated with antibodies against CD105 (SN6, APC-labelled, eBioscience), CD90 and CD73 (5E10 and AD2, respectively, both phycoerythrin (PE)-labelled, BD Bioscience) and podoplanin (purified, kindly provided by Dr. D. Kerjaschki, Clinical Institute for Pathology, Medical University of Vienna, Vienna, Austria) and with allophycocyanin (APC)-labelled, PE-labelled and purified IgG controls (eBioscience and BD Bioscience, respectively) at 4°C for 30 min in the dark. Secondary antibody (PE-labelled, Dianova) for podoplanin analysis was incubated at 4°C for 30 min in the dark. After washing with PBS cells were resuspended in ice-cold PBS/2mM EDTA and analyzed. $CD105^{-/low}$ and $CD105^{high}$ cells were identified and electronically gated on either the FACSAria cell sorter or the CyAn ADP analyzer (Beckman Coulter) after APC excitation at 633 nm. The purity of sorted cells was verified directly after sorting and was >98%.

Where additionally SP was investigated, cells were treated for the SP analysis prior to antibody incubation.

The expression of ABCG2 on the cell surface was verified by FACS analysis as described [26].

MPM xenografts into NOD/SCID mice

NOD/SCID mice were bred and maintained under conditions approved by the Animal Care Committee. Sorted cells were resuspended in Matrigel (Matrigel Basement Membrane Matrix; Becton Dickinson) and injected under the renal capsula of 6 - 8 weeks old mice that were irradiated with 1 Gray one day before injection [27]. Tumor tissues obtained from patients were directly implanted under the renal capsula. The mice were anesthetized with isoflurane while cells were injected. All animals were sacrificed between 13 and 21 weeks post-transplantation. Tumor volume in cm^3 was determined using the formula (length x $width^2$) / 2, where length was the longest axis and width being the measurement at right angles to the length.

Results

Identification and characterization of a side population in the MPM cell line ZL55

In preliminary experiments we established a side population protocol using as an experimental control [28] the leukemia cell line HL60/Dox, which over expresses multidrug resistance proteins allowing the cells to efflux the DNA staining dyes Hoechst 33342 or DyeCycleViolet (DCV). We observed that using DCV, which has the advantage of being excited at non-UV wavelength with an enhanced emission signal compared to Hoechst 33342 [23], resulted in a better separation of SP/NSP (Supplementary Figure 1A). In addition we found that DCV could be used at a concentration (0.5 μM) not toxic to cells (Supplementary Figure 2), allowing to take into account cytotoxicity concerns about this method [29]. This DCV protocol was first applied to breast cancer cell line MCF-7 [30] to provide an additional control for low abundance SP (Supplementary Figure 1B and Table I).

Using the DCV protocol for the MPM cell line ZL55 (gating strategy presented in Supplementary Figure 3), we identified a SP of 2.1 ± 1.9 % (n = 31, mean±SD, Figure 1A and Table I), which was decreased by the drug transporter inhibitor verapamil, indicating that these cells represented the SP cells. To examine whether SP cells could regenerate SP and NSP cells, ZL55 cells were sorted into SP and NSP and further cultured in vitro. Reanalysis of ZL55 SP and NSP in respect to their SP/NSP distribution revealed that ZL55 SP was able to regenerate a SP (2.25 %, n = 2), whereas only NSP (n = 2) was obtained with ZL55 NSP. These data suggest that a SP with self-renewal properties is present in MPM ZL55 cells. In addition the ZL55 SP was enriched with cells expressing the drug transporter ABCG2 at both protein and mRNA level (Figure 1B). To further characterize SP vs NSP derived populations the expression of N-cadherin and mesothelin, which we routinely use to characterize our primary cultures [16], were investigated. Both N-cadherin and mesothelin (Figure 1C, left panel) were not detected in ZL55 SP cells in which a higher relative expression of Sox2 was observed (data not shown). This might indicate that ZL55 SP cells are in a less differentiated state. The relative expression of MDR1 was also increased in the SP fraction (Figure 1D), which is not surprising given the fact that DCV is a substrate of various ABC transporters. Relative expression enrichment was

also observed for ABCC3 but not for ABCC1. All in all, these data indicate that a SP with stem/progenitor cell properties is present in ZL55 cells.

Characterization of SP in additional MPM cell lines, MPM primary cell cultures and in a primary mesothelial cell culture

Beside the mesothelioma cell line ZL55 we investigated the SP distribution in 2 additional MPM cell lines ZL34 and H28, in 3 primary MPM cell cultures SDM96, SDM100 and SDM138, in a xenograft derived primary MPM cell culture SDM103T2 (Figure 1A) and in a primary mesothelial cell culture SDM104. In all these cell lines and primary cell cultures a SP was identified (Table I).

The fact that a SP is also present in the primary mesothelial cell culture SDM104 indicates that a fraction of cells with potential self-renewal capacity is present in cultured normal mesothelium.

SDM103T2 cells were selected for further characterization for two reasons: first, they were derived from xenograft implantation of a tumor after chemotherapy, offering the opportunity to detect chemotherapy resistant cells. Second, tissue of relapsed tumor from the same patient was also available for further comparison. As for ZL55 cells, the SDM103T2 SP (Figure 1B, right panel) showed increased expression of ABCG2 and was able to regenerate a next SP (2.25 %, n = 1) when cultured. The latter (SDM103T2 SP1 SP1, Figure 1B) was even further enriched for ABCG2 expression compared to the remaining NSP (SDM103T2 SP1 NSP1). Similarly to ZL55 cells, the expression of the MPM marker mesothelin was lower in SP compared to NSP (Figure 1C, right panel) while this trend was not observed for N-cadherin. Thus, it seems that SP phenotype is accompanied by decreased expression of mesothelin, which is generally abundantly expressed in epithelioid MPM [31]. No specific enrichment of other ABC transporters in the SP fraction was observed (data not shown).

SP cells tend to be more tumorigenic than NSP cells

To test whether the tumorigenicity of SP and NSP differs, various numbers (10^2 -10^5) of sorted ZL55 cells were injected under the renal capsule of NOD/SCID mice and monitored for tumor development. Both ZL55 SP and NSP gave rise to tumors when 10^5 cells were implanted (n=4). By decreasing the injected cell number below 10^3, neither ZL55 SP nor NSP gave rise to tumors (n=2). Injecting between 10^3 and 10^4 cells of ZL55 SP and NSP led to tumor development for both SP and NSP in three of

four mice tested. Therefore, similar to another study [15] no difference in a cell line SP vs NSP tumorigenicity was observed. We then injected sorted SP and NSP cells derived from a SP (ZL55 SPT) and a NSP (ZL55 NSPT3-3) tumor. Both cell cultures gave rise to a SP fraction (0.9 ± 0.7 % and 0.2 ± 0.1%, n = 8 and n = 3, respectively). By injecting between 10^3 and 10^4 of these tumors' derived, sorted cells, there was a tendency (Figure 2A) to observe tumor formation more frequently with the SP fraction. For the xenografted tumor derived SDM103T2 only SP cells gave rise to tumors. All in all, there is a tendency ($p=0.12$, two-tailed chi-square test) for the SP fraction to have higher tumor initiating ability (8/14) compared to NSP fraction (4/14).
The existence of intrinsic differences between SP and NSP sorted cells was supported by colony forming efficiency (CFE) assay. Plating ZL55 SPT SP and NSP cells resulted in the growth of two colony-types: compact and loose (Supplementary Figure 4). The yield of loose colonies was significantly ($p<0.01$, Mann-Whitney test) higher in ZL55 SPT NSP compared to SP. SDM103T2 could not be analyzed in a similar way because cells grow spreading in the dish. However, a similar observation was obtained in ZL34 cells.

SP derived tumors shift their expression toward mesothelium precursor phenotype

In order to get further insight into the mechanism of increased tumorigenic potential of SP derived cells, gene expression and immunostaining was performed on SP and NSP derived tumors. The relative expression of mesothelioma markers calretinin [32], podoplanin [33] and mesothelin [31]; ABC transporters ABCG2, MDR1 and ABCC1; stem cell markers Sox2, nestin, OCT4A, Bmi-1, CD90; sonic hedgehog activity markers Gli-1, Patch1; hypoxia controlled CAIX and Wisp2 and matrix remodeling Slug, Twist and PAI-1 were investigated in ten ZL55 SP and NSP derived tumors. Some mouse genes (Gli-1, Patch1 and Sca-1) were also included to take into account mouse stromal components. Although of all the genes analyzed only Patch-1 was significantly enriched in SP derived tumors ($p<0.05$, two way ANOVA), it nevertheless indicates that SP and NSP derived tumors are different and suggests the involvement of sonic hedgehog signalling. An unsupervised clustering showed separation of SP and NSP tumors (Figure 2B) with the exception of three tumors. In addition to Patch1, the expression of podoplanin was enriched in SP compared to NSP derived tumors. Latter observation was confirmed by immunostaining of tumor

sections (Figure 2C, 2D and Table II) where we observed that podoplanin staining shifted from the membrane to the cytoplasm in SP derived tumors. It is noteworthy that podoplanin, also called D2-40 antigen, is a marker of splanchnic mesoderm [34] from where mesothelium derives. Hence, SP derived tumors have an increased expression of mesothelium precursor marker.

Mesothelioma is classified according to histopathology into epithelioid, biphasic and sarcomatoid subtype, with the biphasic type being defined as a tumor comprising more than 10% of both epithelioid and sarcomatoid areas [35]. In SP derived tumors we observed a morphological shift from 100% epithelioid tumor to a mixed morphology for ZL55 derived tumors or to an increased spindeloid phenotype for SDM103T2 cells (Table II). The latter was paralleled by a significant decrease ($p<0.01$) in cells with positive nuclear WT1 immunostaining (30% and 98% in ZL55 and SDM103T2 derived tumors, respectively). WT1 is a transcription factor expressed at the time of switch from mesenchymal to epithelial cells (MET) in mesothelium precursor cells [36]. Continuous WT1 expression is conserved in mesothelium through adult life and is maintained in mesothelioma [37]. However, it decreases during epithelial to mesenchymal transition (EMT) occurring for example in sarcomatoid mesothelioma [38]. Altogether these data indicate that SP derived tumors shift their expression toward mesothelium precursor phenotype.

In contrast to observations in vitro where differences in mesothelin and N-cadherin expression were observed in SP and NSP fractions, all tumoral cells showed positive immunostaining for these markers (Supplementary Figure 5). There can be many reasons for it, including growth in an in vivo environment. In addition, all tumors were positive for calretinin.

The resistance of stem-like cells to conventional chemotherapy is thought to be the cause of tumor recurrence. Hence, we investigated whether the recurrence tumor SDM141 from the patient SDM103, from which SDM103T2 were derived, would have a similar shift toward mesothelium precursor phenotype, defined here as the absence of WT1 expression, podoplanin shift from membrane to the cytosol and spindeloid phenotype. We observed indeed a loss of WT1 expression, an increased cytosolic podoplanin immunostaining and a shift toward sarcomatoid morphology in SDM141 sample compared to epithelioid SDM103 phenotype (Figure 2E). These observations indicate first that in the xenograft (SDM103T2) grown in mice, cells selected to grow were already shifting to spindeloid phenotype compared to parental tumor and

second that xenografted SP cells behave like relapsing tumors, buttressing the hypothesis that relapse might be due to resistant cancer stem-like cells. Similar results were observed for tumor evolution in other two patients (Supplementary Figure 6).

ZL55 SPT cells were more chemoresistant to chemotherapeutics

Since mesothelioma patients develop resistance against chemotherapy and because ABCG2 expression is correlated with a higher resistance to chemotherapeutics, we investigated the chemoresistance of ZL55 SPT and ZL55 NSPT3-3 cells. The latter were incubated either with verapamil, with mitoxantrone, a known ABCG2 substrate, or with a combination of verapamil and mitoxantrone (Figure 3A). ZL55 SPT cells were more resistant to mitoxantrone compared to ZL55 NSPT3-3 cells, but this difference was abolished ($p<0.01$, paired *t* test) in the presence of verapamil. Additionally, ZL55 SPT and ZL55 NSPT3-3 cells were incubated with different concentrations of cisplatin, a chemotherapeutic agent commonly used for the treatment of mesothelioma patients. Also in this case, ZL55 SPT cells were significantly ($p<0.001$, t-test) more resistant to a high concentration of cisplatin compared to ZL55 NSPT3-3 cells (Figure 3B). Cisplatin induced DNA damage was similar in both ZL55 SPT and NSPT3-3 cells as indicated by the phosphorylation of the histone variant H2AX (Figure 3C), occurring downstream DNA damage response (DDR) (reviewed in [39]). However, a better survival in ZL55 SPT compared to NSPT3-3 cells was correlated with higher basal expression of survivin (Figure 3C), an antiapototic protein upregulated in MPM [40]. In addition, cisplatin exposure resulted in a further increase of survivin levels which contributes to chemoresistance, as we have previously described [41]. All in all, these data indicate that SP derived cells are more chemoresistant.

SP cells are CD105$^{-/low}$

To gather more information on the SP phenotype we determined whether mesenchymal stem cell markers CD105, CD90 and CD73, which were expressed in all MPM investigated (Supplementary Table II), would be differentially expressed in the SP itself. We also investigated the expression of podoplanin and of ABCG2, which were differentially expressed in the various cultures. The SP was specifically enriched in CD105$^{-/low}$ cells for SDM103T2 (4.9 ± 2.8 fold enrichment, n = 4) (Figure

4A). Similar results were obtained for ZL55 cells. In addition CD105$^{-/low}$ cells were smaller (Figure 4B). No specific enrichment was observed with the other two markers. Sorted ZL55 CD105$^{-/low}$ cells induced tumors which were five times larger ($p<0.02$, $n = 4$, *t*-test) compared to CD105^{high} cells buttressing the existence of a cell subpopulation with high tumorigenic potential (Figure 4C). Consistent with this observation, Ki67 proliferation marker immunostaining was more abundant in ZL55 CD105$^{-/low}$ cells (Figure 4D).

Discussion

In this study we identified a SP with self-renewal and chemoresistance capacity, enriched in CD105$^{-/low}$ cells and able to induce, when implanted under the renal capsule of NOD/SCID mice, the growth of a tumor with increased spindeloid morphology. Furthermore, we observed that CD105$^{-/low}$ cells develop larger tumors compared to CD105^{high} cells. Unfortunately the yield of SP in CD105$^{-/low}$ cells was insufficient to allow any investigation of the double selection. However, based on current knowledge on embryonic mesothelium development, the proposed recruitment of adult precursor cells by asbestos [42] and on our own results we put forward the hypothesis that MPM SP CD105$^{-/low}$ cells include mesothelioma cells responsible for tumor recurrence in patients (Supplementary Figure 7).

MPM SP fractions varied between cell cultures but were in the same range of magnitude as found in other cancer types [43]. They were enriched for ABCG2 transporter expression as observed by others [44], [28] and the fraction of ABCG2 surface positive cells was in a similar range as the SP fraction. In addition the ABCG2 specific inhibitor FTC [45] inhibited dye efflux (data not shown), therefore it is likely that ABCG2 is responsible for the SP phenotype in MPM.

The epithelioid differentiation marker mesothelin was low in the SP fraction. Not much is known about the regulation of mesothelin expression. However in MexTag mice in which the SV40 T-antigen (Tag) is under the control of the mesothelin promoter and which develop MPM tumors upon exposure to asbestos fibres, Tag is not detected in the unexposed mice [46]. This may indicate that the cells that are stimulated to proliferate upon asbestos fibres exposure are undifferentiated precursor

cells. The latter have been recently described in normal mesothelial primary cultures [47].

Similar to another study using MS-1 MPM cell line [15] no difference in tumorigenicity of SP vs NSP was observed, when starting material was a cell line. Nevertheless tumors from xenograft derived SP had a tendency to be more frequent, but most importantly shifted toward a precursor phenotype defined here as enriched for podoplanin expression and low WT1 expression. Consistent with an MET (mesenchymal-epithelial transition) concept and precursors being of mesenchymal phenotype, SP derived tumors shifted backwards from epithelioid to spindeloid morphology characterized by cytoplasmic podoplanin expression as observed by others [33], [48], [49, 50] and decrease of nuclear WT1 staining [38]. This phenotype shift cannot be attributed to the microenvironment, since such changes were not observed in NSP cells derived tumors developed contra laterally. In addition the increase in mesenchymal profile was consistent with the hypothesis that mesothelium injury, due to asbestos fibres accumulation in the pleural space, activates tissue repair involving mesenchymal stem cells (MSC) [42]. It is possible, as suggested by Beachy et al.[4], that persistent injury and stimulation of repair would lead in such cells to oncogenic events, represented in our model by the loss of NF2 and INK4A function (reviewed in [51].

Further, we investigated MSC markers CD105, CD90 and CD73 expression in MPM cell lines and primary cell cultures. All investigated MPM cell lines and primary cell cultures were positive for all three MSC markers but only CD105 was differently expressed in SP and NSP: SP was enriched in $CD105^{-/low}$ cells. The latter developed larger tumors compared to $CD105^{high}$ cells. CD105 is an ancillary transforming growth factor β receptor, and it is not clear whether its differential expression in MPM cells is responsible for the observed phenotype. In hematopoietic stem cells CD105 positive cells contain all the long term repopulating HSC activity within bone marrow SP [52] while others have suggested a function of CD105 as a tumor suppressor in epithelial cancer [53]. Hence, functional studies are necessary to address any role of CD105 in MPM tumorigenicity. Intriguingly $CD105^{-/low}$ cells had also a smaller size. This is in line with observation from Grichnik et al. who identified SP cells in metastatic melanoma cell lines which, compared to NSP cells, were small in size and gave rise to a heterogeneous cell population [54]. A small size for stem-like cells has also been reported in glioma cells [30] and in squamous cell carcinoma (A431) cells [55].

Alternatively to the hypothesis that asbestos fibres recruit and alter a mesothelium precursor, acquisition of oncogenic lesions may occur in terminally differentiated cells resulting in dedifferentiation to a primitive stem-like state. This state is often associated with epithelial to mesenchymal transition and chemoresistance [56] like observed in our study. It would be interesting to further investigate the potential of induction of differentiation as therapeutic option. In this context it is noteworthy that all-trans-retinoic acid treatment decreased growth of a sarcomatoid malignant pleural mesothelioma cell line *in vivo* without induction of apoptosis [57]. Since WT1 is controlled by retinoic acid [58] one possibility which remains unexplored, is that WT1 was induced and it led to tumor cell differentiation and growth arrest.

Taken together our results support the hypothesis that MPM recurrence develops from mesothelium-precursor-like cells. But most importantly we rise for the first time an issue that is seldom taken into account in mesothelioma biology: mesothelium has mesodermal origin and undergoes mesenchymal to epithelial transition during development. Mesothelioma progression, from epithelioid to biphasic to sarcomatoid seems to follow a "backward to precursor" epithelial to mesenchymal transition, which for the moment has not been thoroughly addressed because of the complexity of mesothelium itself which maintains some mesenchymal characteristics (e.g. vimentin expression). Using tools that are already largely used in the clinic such as immunohistochemistry for WT1 and podoplanin we provide knowledge that could be easily implemented in large cohorts of patients to verify whether it could predict time to relapse and allow adaptation of patient follow-up.

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Tables

Table I - Average SP abundance

Cell line		Average SP (%)	number of replicates
Breast cancer*	MCF-7	2.3±0.5	5
MPM	ZL55	2.1±1.9	31
	ZL34	0.4±0.1	3
	H28	0.7±0.3	4
Primary MPM	SDM96	1.1	2
	SDM100	1.1	2
	SDM103T2	1.6±1.5	24
	SDM138	0.2±0.2	3
Primary mesothelial cells	SDM104	2.8	2

NA = not applicable; MPM = mesothelioma; SP = side population; * Used as control [28].

Table II - Spindeloid cells and higher cytoplasmic podoplanin expression in ZL55 SP and SDM103T2 SP derived tumors compared to contra laterally derived NSP tumors or parental cells

	Morphology		Podoplanin		
	Epithelioid	Spindeloid	% positive cells	Mb	Cyt
Mouse 1					
ZL55 **SPT3-3**	90%	5-10%	5	+	++
ZL55 NSPT3-3	100%	0%	2-5	++	+
Mouse 2					
ZL55 SPT **SPT2**	95%	5%	30	++	++
ZL55 SPT NSPT	100%	0%	10	++	+
Mouse 3					
ZL55 SPT **SPT3**	95%	5%	80	+	++
ZL55 SPT NSPT2	100%	0%	30-40	++	+
SDM103T2	20	80	50	++	+
SDM103T2 **SPT**	10	90	10	+	+++
SDM103T2 **SPT2**	0	100	50	+	+++

SPT, side population derived tumor; NSPT, non-side population derived tumor; Mb, membrane; Cyt, cytoplasmic.

Figures

Figure 1.

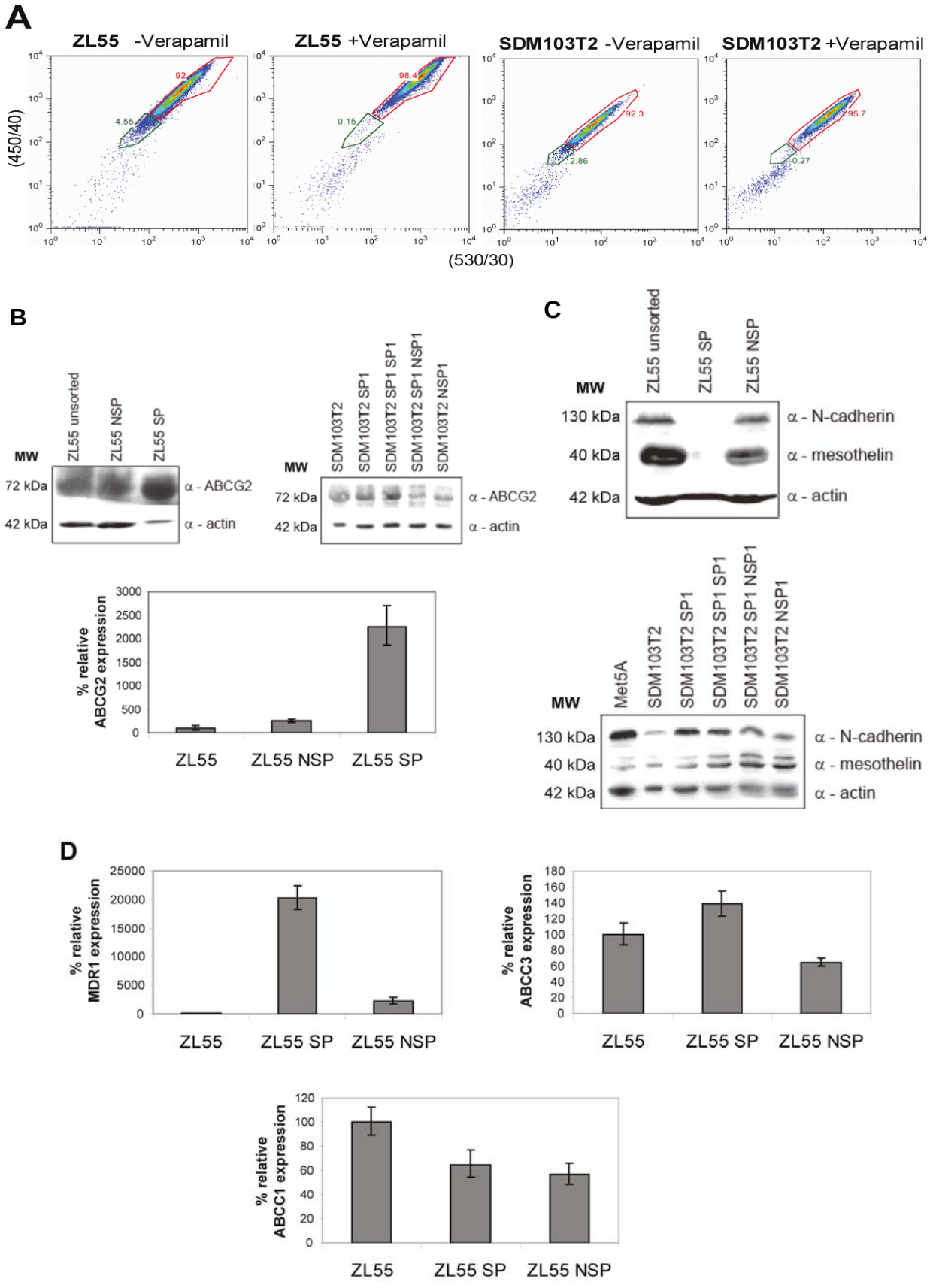


Figure 1 - ABCG2 and mesothelioma markers expression in the side population of a MPM cell line and a primary MPM xenograft cell culture

(A) MPM cell line ZL55 and xenograft SDM103T2 cells were stained with DCV (0.5 μM) in the absence (–) or presence (+) of verapamil (50 μM). The side population (SP, green gate) was defined as the population decreasing by the addition of verapamil. (B) Western blot and real-time PCR analysis to assess ABCG2 expression levels in sorted SP and non-SP (NSP) cells. ABCG2 protein (BXP-21, 1:1000, Alexis, Biochemicals) was elevated in both ZL55 and SDM103T2 SP cells compared to NSP and parental cells. ABCG2 mRNA was also enriched in ZL55 SP cells. (C) Western blot analysis to determine the expression of mesothelin (MN-1, 1:1000, Rockland Inc.) and N-cadherin (32/N, 1:2500, BD Bioscience) mesothelioma markers. Met5A cells were used as positive control for these two mesothelioma differentiation markers. Mesothelin was decreased in exponentially growing sorted SP cells. N-cadherin was also decreased but only in ZL55 SP cells. (D) Real-time PCR analysis revealed higher MDR1 and ABCC3 mRNA expression in ZL55 SP compared to NSP cells. Real-time PCR data were normalized to histones and expressed as mean ± s.d. (n = 3). Actin (C4, 1:10'000, ICN) was used as western blot loading control.

Figure 2.

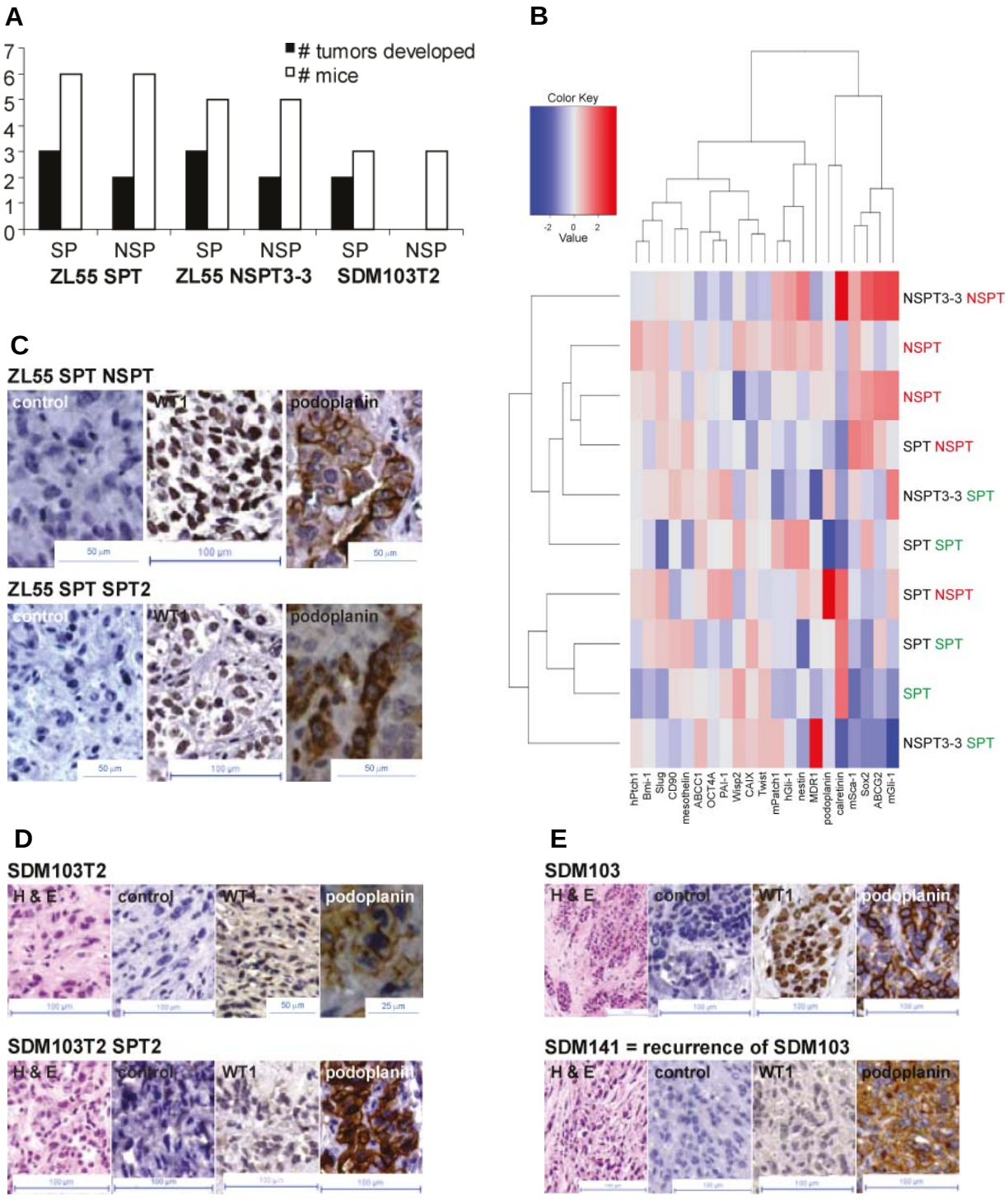


Figure 2 - Different phenotypes in freshly sorted SP/NSP and their derived tumors

(A) SP from xenograft derived tumors were enriched for tumor forming capacity in NOD/SCID mice. 10^3 - 10^4 cells were implanted under the renal capsula of 1 Gy

irradiated mice. (B) Unsupervised clustering of SP and NSP derived tumors. Analyzed genes include ABC transporters ABCG2, MDR1 and ABCC1; stem cell markers Sox2, nestin, OCT4A, Bmi-1, CD90; sonic hedgehog activity markers Gli-1, Patch1; hypoxia controlled CAIX and Wisp2 and matrix remodelling Slug, Twist and PAI-1. Matrix of relative gene expression values is shown as heatmap. Red indicates down-regulated genes, blue indicates up-regulated genes. (C, D) Representative tumors stained with either hematoxylin and eosin (H & E), without primary antibody (control) or with antibodies against WT1 (6F-H2, 1:50, DAKO Cytomation) or podoplanin (D2-40, 1:50, DAKO Cytomation). WT1 expression decreased in SP derived tumor tissues while podoplanin shifted from the membrane to the cytosol compared to NSP derived tumor tissues for ZL55 cells and SDM103T2 tumor. (E) Tumor relapsing in patient SDM103 shifts toward mesothelium precursor phenotype, defined as the absence of WT1 expression, increased podoplanin cytosolic staining and spindeloid phenotype.

Figure 3.

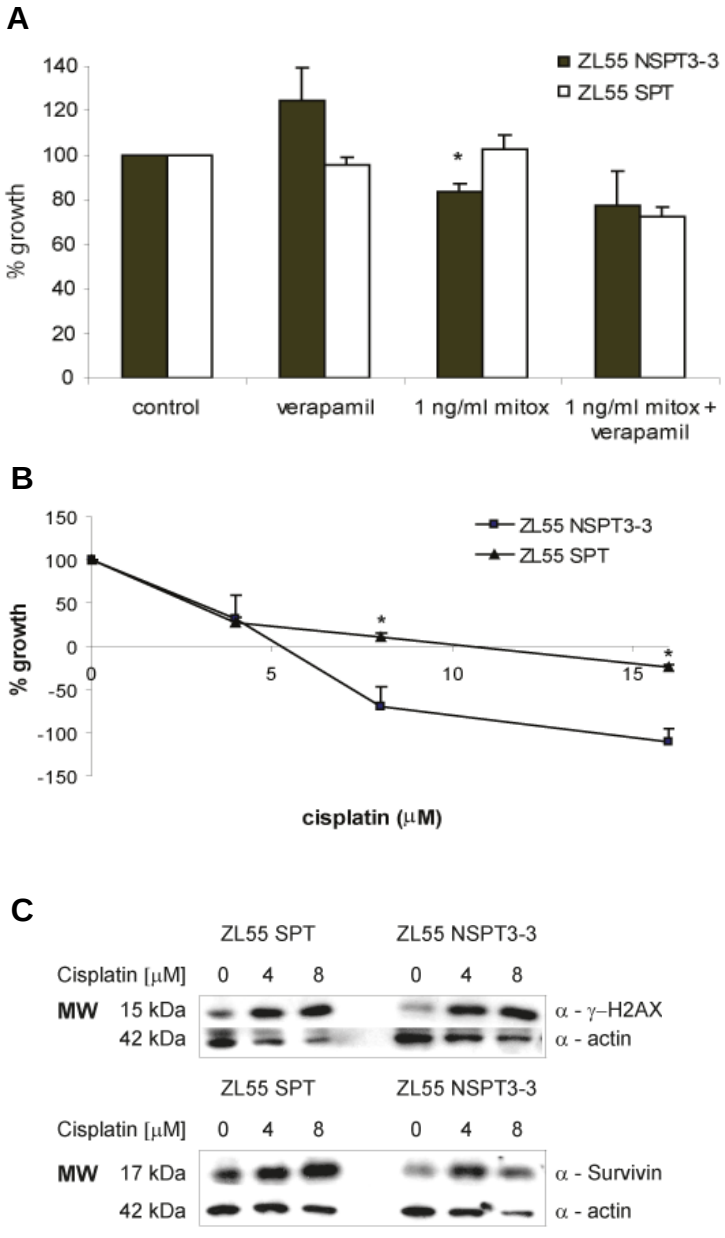


Figure 3 - SP derived tumor cells are more chemore-sistant

(A, B) ZL55 SPT and NSPT3-3 cells were plated in 96-well plates and treated 24h later with verapamil (50 μM), mitoxantrone (1 ng/ml), and a combination of the two for 1h, or with different cisplatin concentrations for 72h. The MTT assay was performed 72h after beginning of the drug treatment. ZL55 SPT derived cells were more (*, $p<0.01$) chemoresistant to mitoxant-rone compared to NSPT3-3 derived cells and this difference was abolished by verapamil. ZL55 SPT cells were also more (*, $p<0.001$) resistant against high doses of cisplatin. The data were normalized to the drug-untreated control.

(C) Western blot analysis to assess the expression of the DNA damage sensing protein γH2AX biomarker (JBW301, 1:1000, Millipore upper panel) and the survival protein survivin (Pab, 1:1000, R&D Systems, lower panel). No difference in the γH2AX biomarker expression between the two lines was detected, however ZL55 SPT cells showed increased survivin expression.

Figure 4.

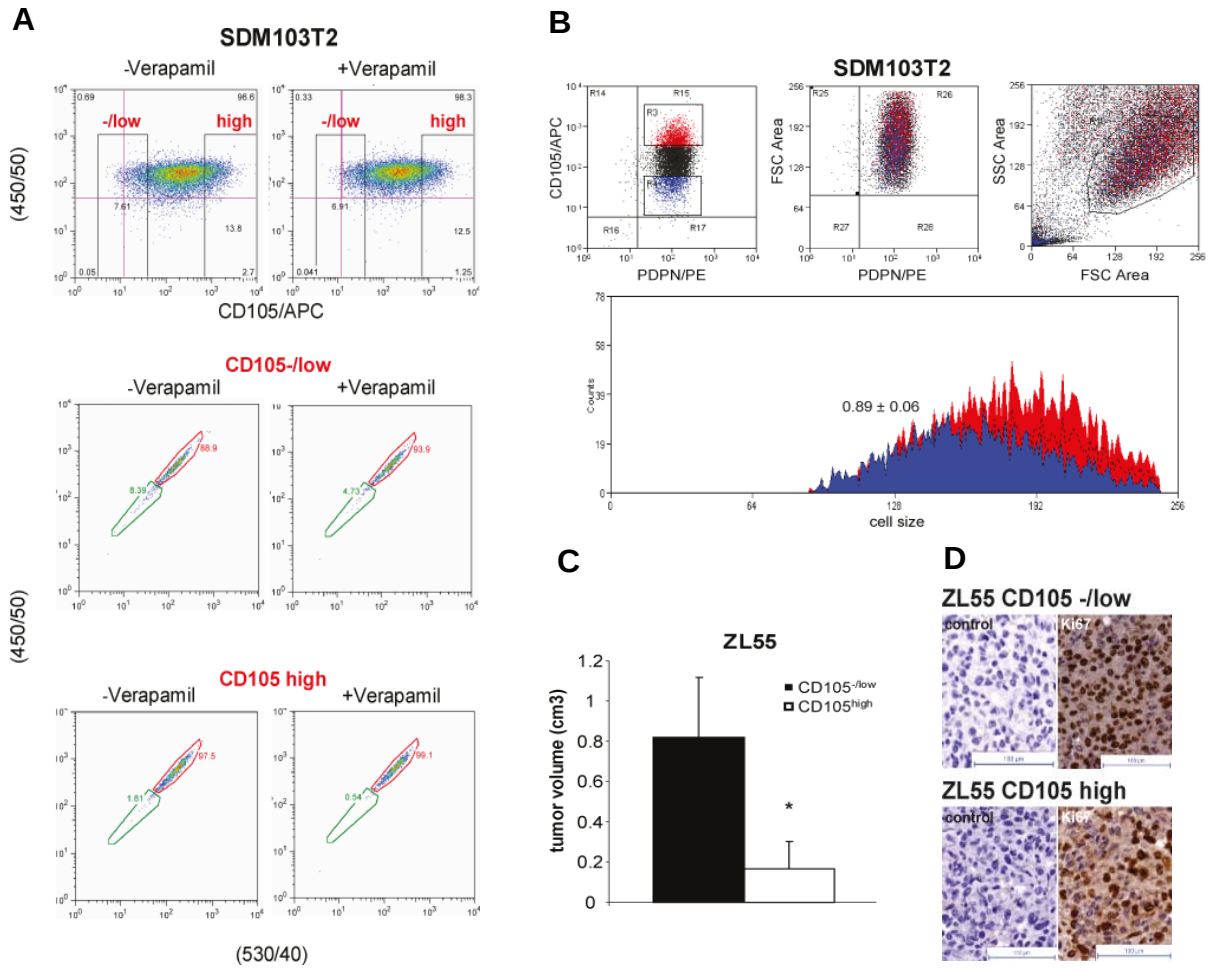


Figure 4 - SP cells are enriched in CD105$^{-/low}$ cells with increased tumorigenicity

(A) The DCV efflux assay was performed to identify SP cells as indicated in the legend to Figure 1, followed by cell staining with an antibody against CD105 labelled with APC (SN6) to identify CD105$^{-/low}$ and ^{high} expressing cells. The SP fraction was then evaluated gating these two populations. (B) SDM103T2 cells were stained with anti-podoplanin and anti-CD105, then CD105$^{-/low}$ and ^{high} cells were analysed in a FSC Area histogram (cell size, lower panel) revealing that CD105$^{-/low}$ cells (blue) are smaller in cell size compared to CD105^{high} cells (red). Number above the cell size plot represents the cell size ratio of CD105$^{-/low}$ to CD105^{high} cells size (n = 9). (C) CD105$^{-/low}$ and high expressing ZL55 cells were sorted, mixed with Matrigel and injected under the renal capsula of 1 Gy irradiated NOD/SCID mice. Tumor volume was assessed after 12 weeks. CD105$^{-/low}$ cells induced significantly (*, p<0.02) bigger

tumors. (D) Increased proliferation in representative CD105$^{-/low}$ and ^{high} derived tumors was buttressed by staining with a proliferation marker Ki67 antibody (B126.1, 1:50, Abcam). No primary antibody was added in controls.

Supplementary Tables

Supplementary Table I Primers used for RT-PCR

gene name	forward primer	reverse primer
mGli-1	GCTGCAACCTTCTTGCTCACACAT	GCTGCAACCTTCTTGCTCACACAT
mPatch1	TGGCCCATGCATTCAGTGAAACAG	TAGGGATCAATGCGGCCATGAAGA
mSca-1	TGGATTCTCAAACAAGGAAAGTAAAGA	ACCCAGGATCTCCATACTTTCAATA

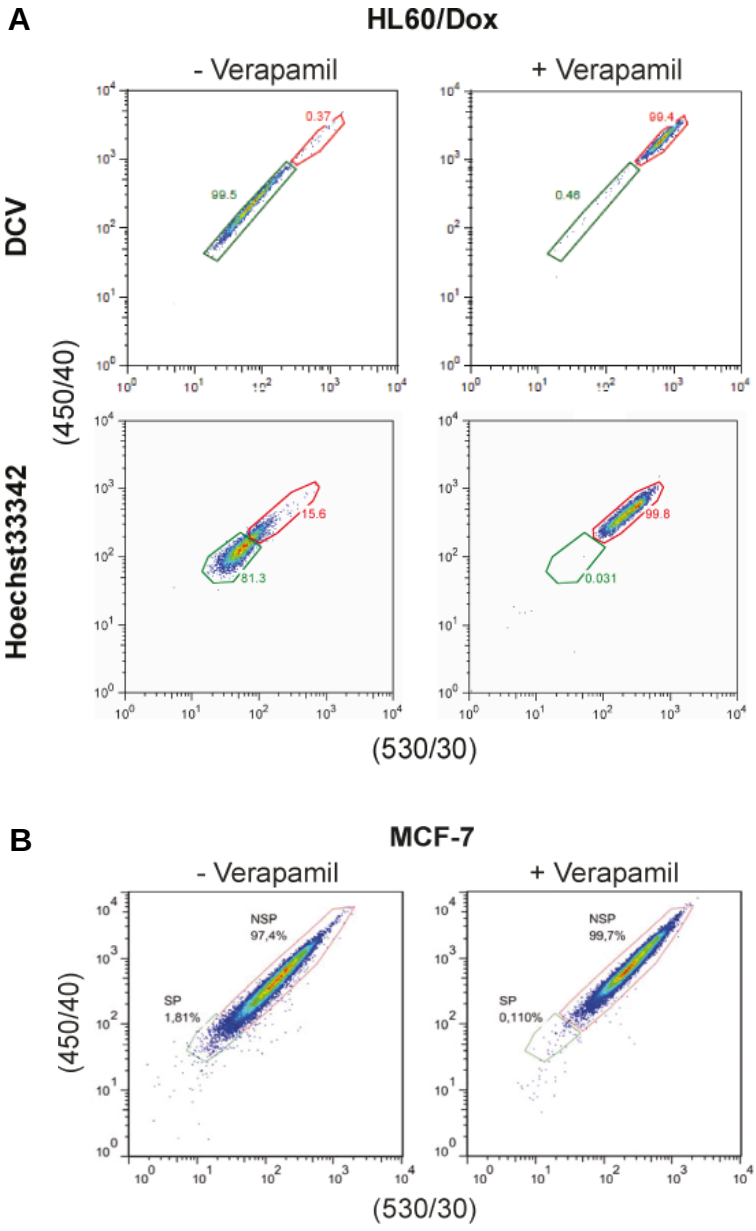
Supplementary Table II - Cell surface antigen expression in mesothelioma cell lines and primary cultures analyzed by flow cytometry

Cell line	CD105	Thy1 (CD90)	CD73	PDPN	ABCG2
ZL55	+++	+++	+++	+	+
ZL55 SPT	+++		+++	+	
ZL55 NSPT3-3	+++			+++	
SDM103T2	+++	+++	+++	+++	+
SDM138	+++	+++			
ZL34	+++	+++	+++	+++	+
H28					+

(+) = 0 – 10% positivity; (++) = 11 – 50 % positivity; (+++) = 51 - 100% positivity

Supplementary Figures

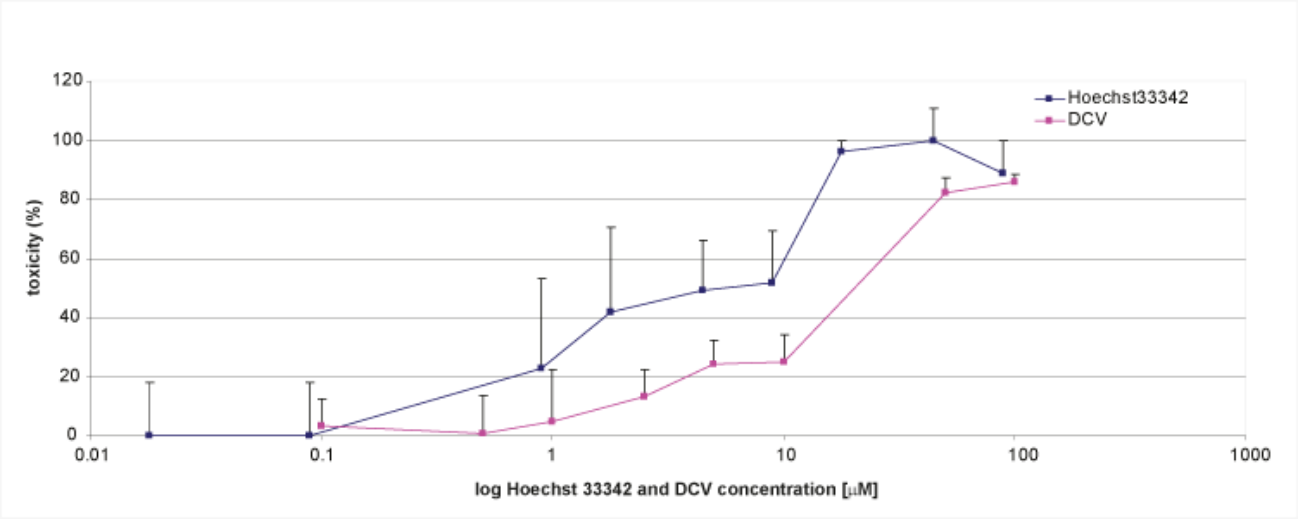
Supplementary Figure 1.



A) The promyelocytic leukemia cell line HL60/Dox selected by chronic exposure to doxorubicin was used with both DyeCycleViolet (DCV) and Hoechst 33342. Verapamil treatment resulted in a better separation of SP/NSP with DCV.

B) MCF-7 cells were used as positive control for low level SP.

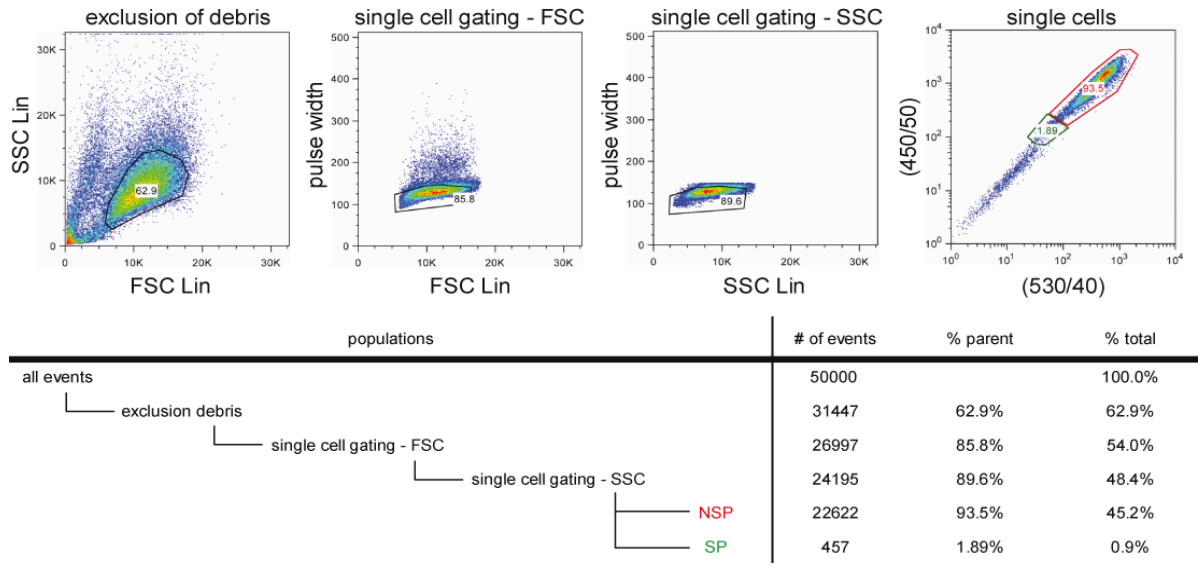
Supplementary Figure 2.



Hoechst 33342 (0 to 50 µg/ml) and DCV (0 to 100 µM) (Sigma – Aldrich) or DCV (Invitrogen) were tested in DMEM/F12+GlutaMax (GIBCO) supplemented with 2% FCS and 10 mM HEPES (GIBCO), for 90 min at 37°C. Then drugs were removed and cells were grown for further 3 days at 37°C before performing an MTT assay to determine cytotoxicity.

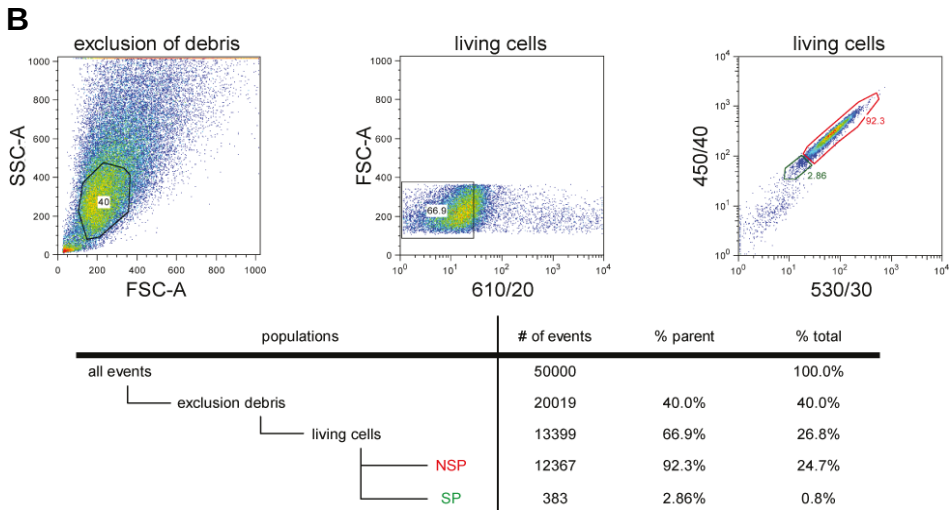
Supplementary Figure 3.

A



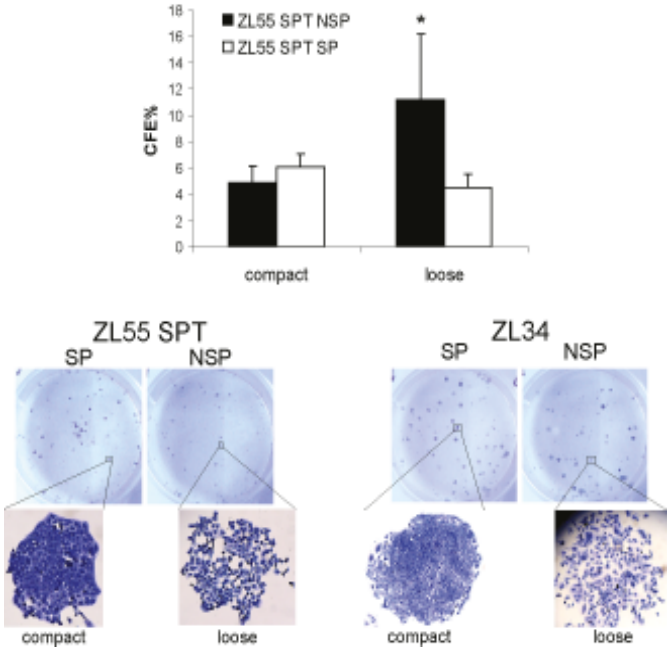
populations	# of events	% parent	% total
all events	50000		100.0%
exclusion debris	31447	62.9%	62.9%
single cell gating - FSC	26997	85.8%	54.0%
single cell gating - SSC	24195	89.6%	48.4%
NSP	22622	93.5%	45.2%
SP	457	1.89%	0.9%

A) Representative gating strategy for single cell setting in cell surface phenotyping



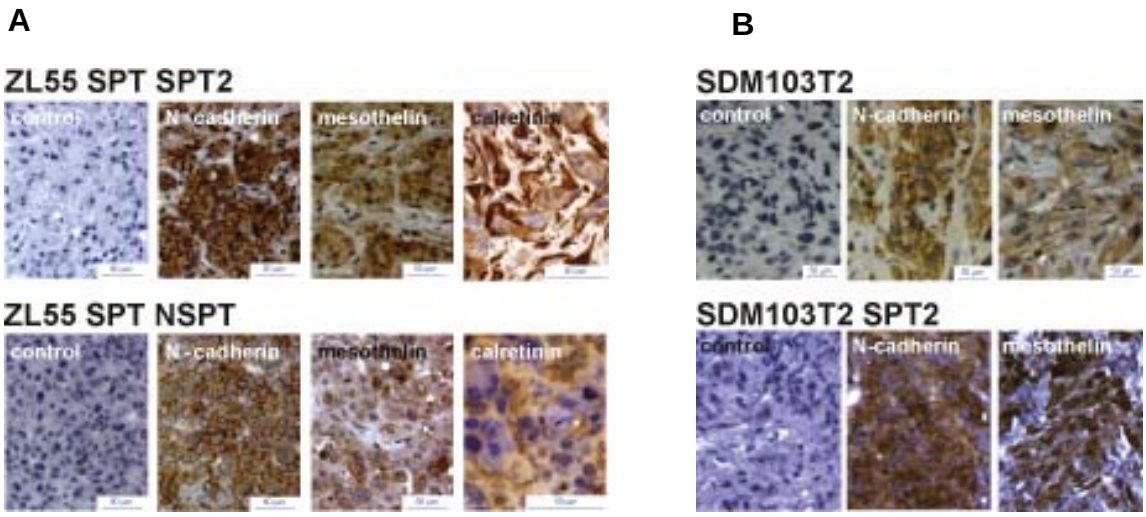
and B) exclusion of dead cells in SP cell sorting

Supplementary Figure 4.



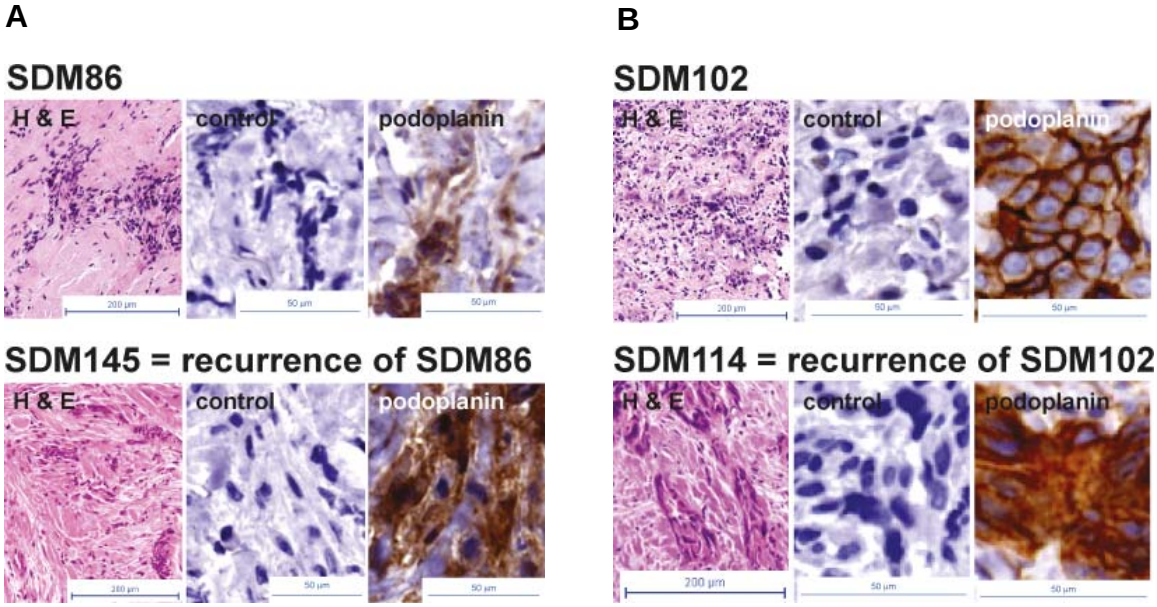
Colony forming efficiency was performed on freshly sorted SP and NSP cells by plating 500 cells/well in 6-well plates. Colonies were stained with crystal violet after 10 days. All colonies with more than 30 cells were counted. Freshly sorted SP and NSP from ZL55 SPT yielded compact and loose colonies and colony forming efficiency (CFE) of loose colonies was significantly ($p<0.01$, $n=6$) higher in NSP fraction. A similar difference was observed for ZL34 cells.

Supplementary Figure 5.



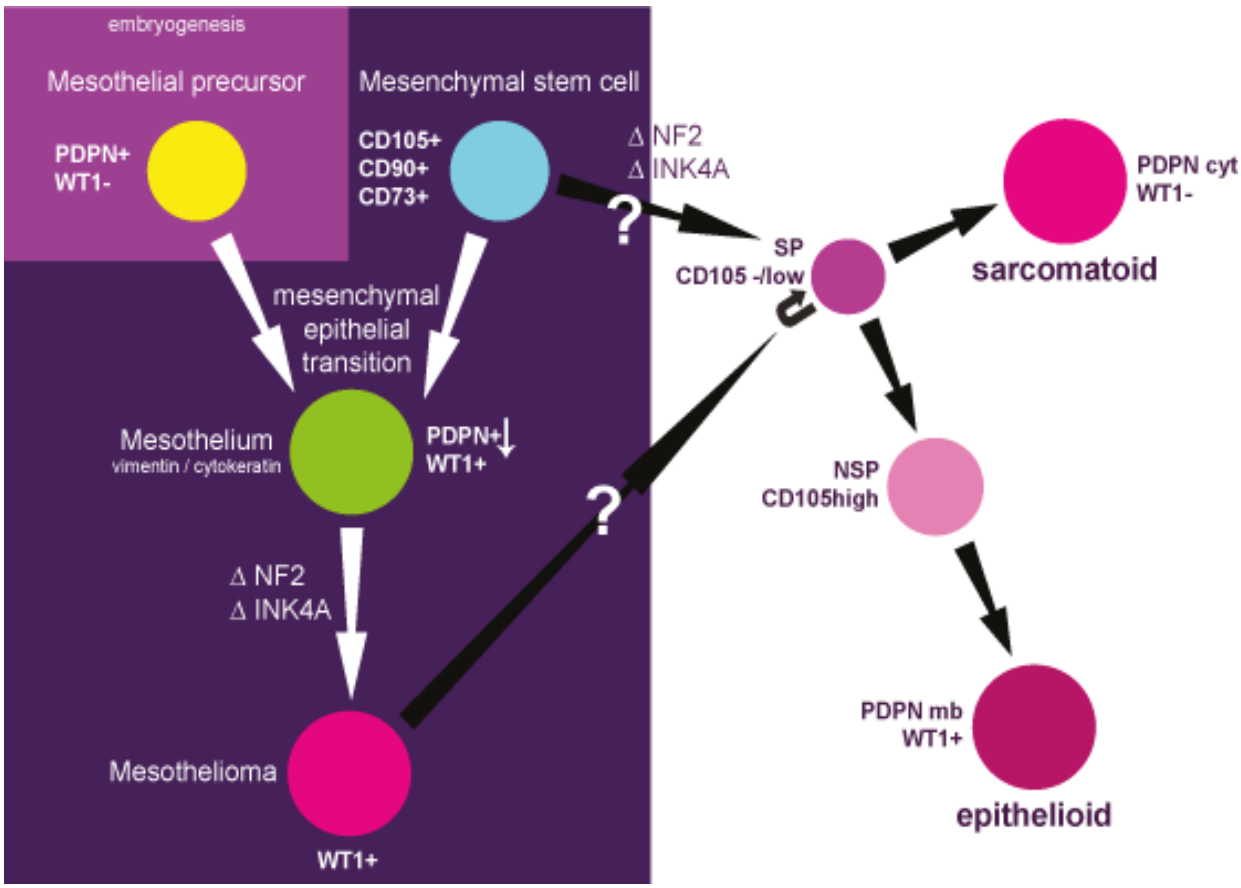
A) ZL55- and B) SDM103T2-derived tumors express N-cadherin, mesothelin and calretinin.

Supplementary Figure 6.



D2-40 immunostaining shifts from membrane to cytosol in recurrence tumors for SDM86 (A) and SDM102 (B).

Supplementary Figure 7.



Upon exposure to asbestos fibres, undifferentiated mesenchymal mesothelium precursor cells or differentiated mesothelial cells undergo oncogenic events (reviewed in [51]) leading to loss of function of NF2 and INK4A tumor suppressor genes and tumor development. $CD105^{-/low}$ SP cells within these tumors are chemoresistant and have higher tumorigenic capacity leading to sarcomatoid evolution of the tumor.

6 DISCUSSION

The aim of this PhD study was to identify and characterize cancer stem cells in malignant pleural mesothelioma. Since no stem cell marker has been yet identified for the mesothelium we used a functional assay.

We observed that SP cells in MPM are accompanied by enrichment in ABCG2 and it can be inhibited by a specific ABCG2 inhibitor. Hence, a legitimate question would be whether it might be worth to sort ABCG2 positive cells, instead of using a functional assay. However, NSP cells do also express ABCG2 as observed at protein and mRNA level although at much lower levels. Therefore sorting ABCG2 positive and negative cells might be difficult to separate. In addition, Patrawala et al. observed that breast, glioma and prostate cancer SP cells were more tumorigenic compared to NSP cells, but $ABCG2^{+}$ and $ABCG2^{-}$ cells, sorted by using the 5D3 antibody which is the only antibody available for sorting living cells, had the same tumorigenic potential *in vivo* (Patrawala et al., 2005). A characteristic of 5D3 antibody is that its binding is ABCG2 conformation dependent which may add difficulty in the interpretation of the results (Ozvegy-Laczka et al., 2005). This additional argument suggests that it is not wise to sort potential cancer stem cells according to ABCG2 surface expression, rather than using the dye efflux assay dependent on ABCG2 function. It is assumed that cancer/tissue stem cells need functional ABCG2 to keep their self-renewal potential. This is supported by the observation of Susanto et al., where ABCG2 function was inhibited by its specific inhibitor FTC leading to decreased Nanog expression (embryonic stem cell marker) in embryonic mouse stem cells (Susanto et al., 2008). Furthermore, the FTC treated cells lost their self-renewal potential (Susanto et al., 2008). In addition overexpression of ABCG2 in murine hematopoietic stem cells kept them in an undifferentiated state (Zhou et al., 2001). Taken together, these findings support the assumption that side populations, dependent on functional ABCG2, harbor cancer/tissue stem cells and functional ABCG2 is needed for their self-renewal potential. Therefore the side population assay is an appropriate method to isolate potential cancer stem cells.

According to the cancer stem cell model, CSCs should induce a phenocopy of the original tumor *in vivo* (Reya et al., 2001). The potential of cancer stem cells to induce tumor growth or their clonogenic potential tested by *in vitro* models like colony formation assays or, where possible, by sphere formation assays represent good supportive experiments and offers the opportunity to test the chemoresistance of cancer stem cells *in vitro*. Nevertheless, they do not take into account possible extracellular factors supporting cancer stem cell functions *in vivo* (Brennan and Matsui, 2009). The capacity of tumor growth *in vivo* is often tested in NOD/SCID mice (the gold standard, (Fillmore and Kuperwasser, 2007), (Jones, 2009)), sometimes irradiated before implantation of selected cells to further suppress a mouse's immune system. For that purpose Quintana et al. used NSG mice to investigate the tumorigenicity of potential melanoma stem cells (Quintana et al., 2008). NSG mice are NOD/SCID mice in which the interleukin-2γ receptor is knocked down leading to a lack of natural killer (NK) cells. Thus, the NSG mice are highly immunocompromised. By comparing tumor growth of transplanted human melanoma cells into NOD/SCID and NSG mice, they observed that tumors grew faster in NSG mice and increased tumor-initiating frequency were observed (Quintana et al., 2008). The *in vivo* system used by Quintana et al. suggesting that in melanoma no cancer stem cells are present, was further investigated by Civenni et al. (Civenni et al., 2011). They sorted melanoma cells according to their CD271 expression and injected them into NOD/SCID, NSG or NOD/SCID mice, in which NK cells were depleted. Comparing tumorigenicity of $CD271^+$ and $CD271^-$ melanoma cells in the different mouse models and investigating the heterogeneity of newly developed tumors in these mice revealed that first of all $CD271^+$ cells represent melanoma stem cells with self-renewal potency and multipotent capacity. Contrary to tumors developed from $CD271^+$ in NOD/SCID, NSG mice developed tumors different from the parental tumors suggesting a role for NK in allowing phenocopying parental tumors as it would be expected from stem cells (Civenni et al., 2011).

In the NOD/SCID mouse model used in this study, we observed in the first tumor generation no difference in tumorigenic potential between freshly sorted SP and NSP cells of a MPM cell line. Tumors developed from second round SP and NSP cells showed a tendency toward increased tumorigenicity of SP cells compared to NSP cells. This observed tendency is in line with the observation done by Roesch et al. (Roesch et al., 2010). They investigated JARID1B (H3K4 demethylase) expressing

melanoma cells with the potential to be cancer stem cells. Serial transplantation assays were used to investigate the long-term tumorigenic potential of JARID1B positive and negative cells during three passages and observed that JARID1B negative cells exhausted their tumor initiating capacity (Roesch et al., 2010). Also Civenni et al. observed long-term tumorigenic potential of $CD271^+$ melanoma cells over five passages (Civenni et al., 2011). This suggests that NSP cells of MPM could lose their tumor growth potential after more than three passages *in vivo*, whereas SP cells should keep their tumor initiating potential *in vivo*, the tendency we already observed. For this purpose, more *in vivo* experiments would have been needed.

However, injection into immunocompromised mice can also be questioned. An ideal solution to overcome this problem would be the usage of a syngeneic *in vivo* mouse model. In such a model, tumor cells and potential cancer stem cells of a mouse tumor can be injected into healthy syngeneic mice. Jongsma et al. developed a mouse model in which mice developed mesothelioma within some hundreds of days (Jongsma et al., 2008). They used the Cre-*Lox*P system to conditionally knock out *Nf2*, *Trp53*, *Rb* and/or *Ink4a/Arf* tumor suppressor genes (Jongsma et al., 2008). These genes have been found to mostly be inactivated/deleted in mesothelioma (see introduction 3.1.7). Differential tumor initiating properties of SP vs. NSP cells derived from tumors of these mice could be investigated to evaluate the role of the immune system during tumor development.

Moreover, the SP fraction of MPM was enriched for tumor initiating cells and we have some evidences that in MPM this function is due to ABCG2. Therefore it might be appropriate to investigate therapies which abolish the function of ABCG2. To be active ABCG2 has to be inserted into the cell membrane (Bleau et al., 2009), (Mogi et al., 2003) and this process is regulated by PI3K/Akt signaling. Mogi et al. showed that indeed PI3K/Akt modulates ABCG2 cell membrane insertion in murine bone marrow cells (Mogi et al., 2003). Further, the treatment of T-cell acute lymphoblastic leukemia using NVP-BEZ235, a PI3K/Akt/mTOR inhibitor which is already in clinical trials (Maira et al., 2008), synergized conventional chemotherapeutics (Chiarini et al., 2010). In addition, they showed that NVP-BEZ235 targeted SP cells by decreasing its fraction and ABCG2 cell membrane expression/insertion.

Beside its ABCG2 membrane insertion, PI3K/Akt pathway may additionally be involved in mesothelioma progression (Opitz et al., 2008). Indeed, the decrease of a well-known endogenous inhibitor of the PI3K/Akt pathway, phosphatase and tensin homologue (PTEN) was associated with a worst outcome for patients.

In our study we additionally observed a “backward to precursor” transition as mesothelioma progresses from epithelioid via biphasic to sarcomatoid histotype in the three patients investigated. Due to this observation and the increased potential of SP cells to induce the growth of tumors containing spindelioid cells, we hypothesize that MPM SP cells are responsible for recurrence in patients. Since we analyzed relapse samples from only three patients, this observation will need to be corroborated in a bigger cohort of patients.

The question remains if SP cells are transformed mesothelial stem cells or if they dedifferentiated from tumor cells which re-acquired stem-like properties as recently proposed (Gupta et al., 2009).

It had been hypothesized that after injury mesothelium is repaired through recruitment of submesothelial mesenchymal precursors or bone marrow-derived circulating precursors, possibly, mesenchymal stem cells (Mutsaers et al., 2007). In a surfaceome screen performed in our laboratory, the expression of two well established mesenchymal stem cell markers (MSC) (Dominici et al., 2006), CD90 (Thy1) and CD73, were observed to be expressed on mesothelioma cells (Ziegler et al., manuscript submitted). The expression of CD90 on mesothelioma cells was confirmed in a recent publication (Melotti et al., 2010). These observations together with the possible role of MSC in mesothelium repair as we would expect after asbestos injury, lead us to investigate the expression of the third essential MSC marker CD105 (endoglin) (Dominici et al., 2006). We surprisingly observed that all MPM cells *in vitro* were positive for those markers.

Since MSC markers on MPM cells were detected *in vitro* it would be interesting to investigate if and to what percentage these MSC markers are expressed in MPM *in vivo*. This has already been tested for CD90 immunoreactivity on frozen samples and only few mesothelioma cells were positive (Ziegler et al., manuscript submitted). This *in vitro* vs. *in vivo* discrepancy warrants further investigations. The other two markers still need to be investigated *in vivo*. If *in vivo* mesothelioma cells with expressing

MSC markers are indeed rare, it would be interesting to investigate whether sorted MSC marker positive MPM cells would have higher tumor initiating capacity in NOD/SCID mice. In addition, it would be interesting to investigate differentiation potential of freshly isolated MSC marker positive MPM into osteoblast-, adipocyte- and chondrocyte-like cells. It would support the notion that multipotentiality is maintained in cancer and would provide potential perspective for therapeutic intervention. This would be especially important, if MSC positive cells would have higher tumor initiating capacity.

As described in the results part, MPM SP cells were enriched for $CD105^{-/low}$ cells. CD105 was the only one of the three MSC markers which has a wide range of expression levels in MPM and $CD105^{-/low}$ cells were more tumorigenic which raises the question what function CD105 could have in mesothelioma development/biology.

As mentioned in the manuscript (results part), CD105 is an accessory TGF-β receptor expressed mostly but not exclusively on vascular endothelial cells (reviewed by (Lopez-Novoa and Bernabeu, 2010)). Its expression is low in resting endothelial cells (reviewed by (Lopez-Novoa and Bernabeu, 2010)) but high in proliferating vascular endothelial cells (reviewed by (ten Dijke et al., 2008)). Further, CD105 has a pivotal role in vascularization and CD105-null mice die by the age of E10.5 (Arthur et al., 2000). In human embryogenesis CD105 expression on mesoderm-derived vascular endothelium is observed during the development of the cardiovascular system starting from week 4 onward (reviewed by (Lopez-Novoa and Bernabeu, 2010)). In a prostate carcinoma cell line CD105 down-regulation was observed together with malignant progression (Lopez-Novoa and Bernabeu, 2010) and CD105 overexpression in esophageal squamous cell carcinomas reduced its tumorigenicity (Lopez-Novoa and Bernabeu, 2010). Since CD105 is a receptor for TGF-β a possible question is whether TGF-β modulates MPM growth. TGF-β has ambiguous functions: on one hand it has tumor suppressive and on the other hand pro-oncogenic activities (reviewed by (Roberts and Wakefield, 2003)). Its activity depends on the tumorigenic stage at which it plays a role. In the premalignant stage it has a tumor suppressor function (Roberts and Wakefield, 2003). This switches to pro-oncogenic at later stages which lead to tumor invasion and metastasis (Roberts and Wakefield, 2003).

TGF-β treatment of mesothelial cells *in vitro* induced EMT (Mutsaers and Wilkosz, 2007). Overexpression of TGF-β has been observed in MPM tumor cells often correlated with a poor prognosis (summarized by (Suzuki et al., 2007)).

If $CD105^{-/low}$ cells grow faster *in vivo* compared to $CD105^{high}$ cells it would be assumed that TGF-β has a negative regulatory role. However ATRA which has been used to efficiently arrest the growth of MPM *in vivo* was shown to decrease TGF-β (Tabata et al., 2009) which may suggest that TGF-β is a growth stimulant.

An additional point which would deserve to be addressed is whether SP cells would be WT1 negative, since WT1 is a marker for fetal and adult human mesothelial tissues (Amin et al., 1995) and sarcomatoid MPM loose WT1 expression. Assuming SP cells are $WT1^-$ and NSP cells $WT1^+$, this would be indicative that indeed SP cells dedifferentiated from MPM $WT1^+$ cells towards mesothelial precursor cells. On the other hand, assuming SP cells are $WT1^+$, what would be the regulating mechanism decreasing WT1 expression of SP cells *in vivo*? It is known that WT1 expression is lost upon EMT (Bax et al., 2011), but the mechanism regulating WT1 expression is still unknown. Initially, WT1 was thought to function as tumor suppressor gene, but in recent years evidences suggested an oncogenic function of WT1 in some cell lineages (reviewed by (Hohenstein and Hastie, 2006)). Indeed, in some cases such as Wilms' tumor loosing WT1 has oncogenic effects (reviewed by (Hohenstein and Hastie, 2006)). WT1 expressing adult tumors are assumed to be mostly of epithelial origin and progress via EMT to tumors with worst prognosis (reviewed by (Hohenstein and Hastie, 2006)).

Going into a similar direction, it would be interesting to investigate the function of podoplanin during MPM progression, because podoplanin expression shifted from the cell membrane towards the cytoplasm as MPM progressed from epithelioid to sarcomatoid histotype (results part). This phenomenon has already been observed in clinical samples (Padgett et al., 2008) but it has not been further investigated and diagnostic pathologists score only membranous podoplanin at the moment.

During embryogenesis podoplanin is expressed in different tissues which are well separated in time and space (Gittenberger-de Groot et al., 2007). They include the coelomic cavity epithelium (investigated by immunohistochemistry of mouse embryos

using antipodoplanin clone 8.1.1) (Gittenberger-de Groot et al., 2007), splanchnic mesoderm which is the embryonic origin of the pleura (Gittenberger-de Groot et al., 2007), (Mahtab et al., 2008), osteoblasts (investigated by immunohistochemistry of rat tissues using antibody clone E11) (Wetterwald et al., 1996) which originates from MSC and in lymphatic endothelium where it is under the control of the lymphatic endothelial cell determining *Prox1* gene (summarized by (Schacht et al., 2003)).

Podoplanin is a transmembrane protein with an extracellular, transmembrane and cytoplasmic domain. It was shown that its cytoplasmic domain is necessary to induce EMT via activation of RhoA, a member of the Rho GTPase family involved in cytoskeletal rearrangements (summarized by (Martin-Villar et al., 2006)). Further, it was shown, that podoplanin binds with its cytoplasmic domain to members of the ERM (ezrin, radixin, meosin) protein family, which links integral membrane proteins to the actin cytoskeleton (Martin-Villar et al., 2006). Additionally, they showed that neither deletion of the extracellular domain nor mutations of the cytoplasmic cytoskeleton binding domain or its deletion influenced podoplanin localization at the cell surface (Martin-Villar et al., 2006). On the other hand, Fernández-Muñoz et al. observed the requirement of the transmembrane and cytoplasmic domain of podoplanin to be localized at lipid rafts of the cell membrane (Fernandez-Munoz et al., 2011). The lack of its insertion into these membrane structures inhibited EMT (Fernandez-Munoz et al., 2011). It is generally assumed, that podoplanin plays a role in tumor cell invasion, migration and metastasis, which are hallmarks of malignant tumors (Wicki and Christofori, 2007). Furthermore, it has been suggested, that podoplanin induces tumor invasion without E- to N-cadherin switch, an EMT characteristic (reviewed by (Wicki and Christofori, 2007)).

In our study, MPM cells of spindeloid morphology had increased podoplanin expression compared to cells of epithelioid morphology. As Martin-Villar et al. suggested, podoplanin might be involved in tumor progression (summarized by (Martin-Villar et al., 2006)). Podoplanin expression in mesothelial precursor tissues supports the necessity to investigate the tumor progression capacity of podoplanin positive MPM cells.

7 CONCLUSION

The data obtained in this study provide evidence for a role of SP cells in mesothelioma progression and suggest that SP cells resemble mesothelial precursor cells.

The identification of the mechanism regulating the function of ABCG2 responsible for the SP phenotype will allow targeted chemotherapy in combination with cisplatin/pemetrexed in order to not only erase cancer cells but also cells responsible for relapse and chemoresistance.

The data presented in this study add knowledge about MPM biology and identify potential new interesting markers of tumor progression.

8 FUTURE PERSPECTIVE

Our work may inspire the screening of a large cohort of patients for the histological evolution of epithelioid MPM to evaluate the validity of the model of epithelial to sarcomatoid progression. We have provided some markers, membranous podoplanin and nuclear WT1 as tools for early EMT assessment. These markers are already used in pathology, so this would not be difficult to implement. This study would be feasible within a network of centers (e.g. the recently founded European Thoracic Oncology Plateform) collecting biopsies for diagnostic purposes and again at surgery. Although patients undergoing surgery represent only a minority of MPM patients, it should be possible to reach within such structure enough clinical samples to be informative. If such a study would confirm the hypothesis it would add a significant argument in favour of EMT existence in tumor progression. This has been recently challenged in this year's Association for Cancer Research (AACR) meeting, where the pathologist David Tarin questioned the EMT supposed to be responsible for invasion and metastasizing simply because this process has never been observed in action in human cancers (Ledford, 2011).

In general, it is a big issue to improve malignant pleural mesothelioma treatment due to reasons mentioned in this study. Prevention represents a real advancement for the protection of the population against asbestos fibers and one step into this direction

was done, when the use of it was banned. Unfortunately, this is not true for all parts of the world. Especially in third world it still might be in use or its elimination and deposit occurred improperly. MPM incidence still rises in Europe where asbestos has been banned and in the developing countries not much is known, but it is likely that incidence is rising there as well and it will not decrease so soon ([Anon], 2010). The potential danger of MPM caused by carbon nanofibers which have the same physical properties as asbestos fibers is only now beginning to be evaluated (Donaldson et al., 2010), (Poland et al., 2008). Due to the difficulties in the diagnosis of MPM, early detection and preventing its malignant progression might represent a way to improve the outcome. In this respect MPM biology and its malignant progression needs to be better understood.

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APPENDIX 1

IMIG TNM staging system. Adapted from Moore, 2008.

Primary tumour (T)	
T1a	Tumour limited to the ipsilateral parietal including mediastinal and diaphragmatic pleura, no involvement of the visceral pleura
T1b	Tumour involving the ipsilateral parietal including mediastinal and diaphragmatic pleura, scattered foci of tumour also involving the visceral pleura
T2	Tumour involving each of the ipsilateral pleural surfaces (parietal, mediastinal, diaphragmatic and visceral pleura) with at least one of the following features: • involvement of diaphragmatic muscle • confluent visceral pleural tumour (including the fissures) • extension of tumour from visceral pleura into the underlying pulmonary parenchyma
T3	Locally advanced but potentially resectable tumour; tumour involving all of the ipsilateral pleural surfaces (parietal, mediastinal, diaphragmatic and visceral pleura) with at least one of the following features: • involvement of the endothoracic fascia • extension into the mediastinal fat • solitary, completely resectable focus of tumour extending into the soft tissues of the chest wall • non transmural involvement of the pericardium
T4	Locally advanced technically unresectable tumour; tumour involving all of the ipsilateral pleural surfaces (parietal, mediastinal, diaphragmatic and visceral) with at least one of the following features: • diffuse extension or multifocal masses of tumour in the chest wall with or without associated rib destruction • direct transdiaphragmatic extension of tumour to the peritoneum • direct extension of tumour to the contralateral pleura • direct extension of tumour to one or more mediastinal organs • direct extension of tumour into the spine: tumour extending through to the internal surface of the pericardium with or without a pericardial effusion • tumour involving the myocardium
Lymph nodes (N)	
Nx	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastases
N1	Metastases in the ipsilateral bronchopulmonary or hilar lymph nodes
N2	Metastases in the subcarinal or the ipsilateral mediastinal lymph nodes including the ipsilateral internal mammary nodes
N3	Metastases in the contralateral mediastinal, contralateral internal mammary, ipsilateral or contralateral supraclavicular lymph nodes
Metastases (M)	
Mx	Presence of distant metastases cannot be assessed
M0	No distant metastasis
M1	Distant metastasis present

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11 CURRICULUM VITAE

Curriculum Vitae

Claudia Frei

Culmannstrasse 59
8006 Zürich
Switzerland
Mobile: 0041-79-446 69 73
Email: unnar@gmx.ch

EDUCATION:	July 2011	**PhD** (Dr. sc. nat.) degree in Tumorbiology, University of Zurich, Switzerland
	March 2007	**Diploma** (MSc) degree in Biochemistry, ETH Zurich, Switzerland
	November 2004	**Second Pre Diploma** in Biochemistry, ETH Zurich, Switzerland
	April 2003	**First Pre Diploma** in Biochemistry, ETH Zurich, Switzerland
	January 2001	**Matura** in Zurich, Switzerland
	1996 – 2001	**High School** in Zurich, Switzerland
	1988 – 1996	**Primary School** in Stäfa, Switzerland
RESEARCH EXPERIENCE:	Nov 2007 – July 2011	**PhD project** in the lab of Prof. Dr. med. Rolf A. Stahel, Laboratory for Molecular Oncology, Clinik for Oncology, University Hospital Zurich, Switzerland "Identification of cells with stem cell / self renewal properties in malignant pleural mesothelioma"
	Jul – Sept 2007	**Traineeship** in the lab of Dr. Kathryn M Stowell, Institute of Molecular Biosciences, Massey University, Palmerston North, New Zealand "MGMT in melanoma/glioma cell lines"

	Jan – Jul 2006	**Diploma Thesis** in the lab of Prof. Matthias Peter, Institute of Biochemistry, ETH Zurich, Switzerland "Characterization of novel substrates and subunits of the human Cul3-ubiquitin E3 ligase"

BACKGROUND COURSES INCLUDE:

Practical Courses:

Semester work in the lab of Prof. Matthias Peter, Institute of Biochemistry, ETH Zurich, Switzerland (Oct – Dec 2005)
"Investigation of Rabring7 and RuvBL1 / RuvBL2 as possible subunits of the Cul3-complex"

Practical training in Immunology (2005), supervised by Prof. Hans Hengartner, Immunology, ETH Zurich, Switzerland

Experimental Biology I and II (2004 / 05), ETH Zurich

Lecture Courses:

Toxicology (Cancer, general and environmental Toxicology, applied Ecotoxicology)

Cell Biology (Organogenesis, Growth factors)

Biochemistry I and II

Immunology I and II

Genetics (Gene and Protein Technology, microbial genetics, molecular genetics of eukaryotes)

PUBLICATIONS:

2011

Frei C., Opitz I., Soltermann A., Fischer B., Moura U., Rehrauer H., Weder W., Stahel R. and Felley-Bosco E.
"Pleural mesothelioma side populations have a precursor phenotype"
Carcinogenesis, in press

June 2007

Sumara I., Quadroni M., **Frei C**., Olma M., Sumara G., Ricci R., and M. Peter (2007)
"A Cul3-based E3 ligase removes Aurora B from mitotic chromosomes, regulating mitotic progression and completion of cytokinesis in human cells"
Developmental Cell 12, 887 – 900

EMPLOYMENT:	Apr – Jun 2007	Assistant in Biochemistry in the lab of Prof. Matthias Peter, Institute of Biochemistry, ETH Zurich, Switzerland (level of employment: 100%)
	Jul 2006 – Mar 2007	Laboratory technician in the lab of Prof. Matthias Peter, Institute of Biochemistry, ETH Zurich, Switzerland (level of employment: 20%)
	Jul 2005 – Sept 2005	Laboratory technician in the lab of Prof. Matthias Peter (level of employment: 50%)
	June 2005	Laboratory technician in the lab of Prof. Matthias Peter (level of employment: 20%)
	1999 - 2003	Different secretarial jobs

SKILLS AND QUALIFICATIONS:

Languages:	German (first language)
	English (fluent, Certificate in English, Language International, Auckland, New Zealand, Mar 2001 – May 2001)
	French (high school diploma, 1996 – 2001)
	Latin (high school diploma, 1996 – 2001)
Computer:	MS Office (Word, Excel, PowerPoint) Adobe (Photoshop and Illustrator) OpenLab FlowJo

AREAS OF INTEREST:

Horse riding, Volleyball (key player), Beachvolleyball, Skiing

Travelling, photography, cooking

Member of the organisation committee of the Icelandic horse tournament “Tölt at Dolder” (www.eistoelt.ch)